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Biogeochemistry of Photosymbiosis in Host Tissues and Skeletons of the Species *Tridacna*

Part 1 of 2

A thesis submitted for the degree of Doctor of Philosophy

November 1999

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Abstract

Algal/invertebrate endosymbioses are common in the marine environment and appear to be nutritional in nature. The giant Tridacnid clams form a mutualistic extracellular endosymbiosis with dinoflagellate algae (zooxanthellae) of the species *Symbiodinium microadriaticum*. The large size of the Tridacnids is generally attributed to the nutritional role of their endosymbionts. This thesis examines the nature and mechanisms by which important biochemicals, such as lipids, are translocated from the symbionts to the host.

Methodologies have been developed to enable the determination of the carbon isotope composition of individual saturated and polyunsaturated fatty acids to enable natural abundance isotopic variations in these compounds to be investigated in the Tridacnid-algal symbiosis.

Compound specific isotope analysis of fatty acids in different species of Tridacnids, their zooxanthellae and non-symbiotic species reveal that several fatty acids are apparently directly translocated from algae to host. Evidence is presented for the synthesis of fatty acids from acetate by the clam. Carbon isotopic data also reveal that carbon limitation may play a role in lipid metabolism in giant clams.

Compound specific isotope analysis has also been used to investigate the contribution of translocated compounds during a diel cycle in clam haemolymph and reveals that concentrations of key metabolites vary over the diel cycle as a function of irradiance. The cyclic sugar alcohol scyllo-inositol was detected in clam haemolymph, which may be related to osmoregulation in the clam or to a signalling role in cell proliferation.

Oxygen and carbon stable isotope compositions and strontium abundances of shell carbonate are examined in several clams subjected to varying degrees of phosphate and ammonium nutrient supplementation to investigate the relationship between growth rate and stable isotope compositions and strontium contents.

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For Iain, Daniel and Paul
and
most of all myself!

"Give me books, fruit, french wine and fine weather
and a little music out of doors, played by somebody I do not know."

-John Keats, 1819

"This brilliant expanse, several miles in width, is on all sides divided, either by a line of snow-white breakers from the dark heaving waters of the ocean, or from the blue vault of heaven by the strips of land, crowned by the level tops of the cocoa-nut trees. As a white cloud here and there affords a pleasing contrast with the azure sky, so in the lagoon, bands of living coral darken the emerald green water."

Charles Darwin, 1845, *Journal of researches into the Natural History and Geology of the Countries visited during the Voyage round the World of H.M.S. 'Beagle' under command of Captain Fitz Roy, R.N.*

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1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Present day coral reefs are tropical shallow water ecosystems, which are largely restricted to the area between latitudes 30°N and 30°S (Fig. 1.1). An accurate estimation of the extent of coral reefs in the world is hard to determine although Smith (1978) calculated that living coral reefs cover about 15% of the world's seabed in shallow to 30m water depths. The Great Barrier Reef (GBR) is the largest system of coral reefs in the world (Fig. 1.2) and one of the richest marine habitats on Earth. Approximately 70% of the hermatypic corals (>300 species) present in the Indo-Pacific region are found along the eastern coast of Australia (Wells and Jenkins, 1988).

Modern tropical seas have very low standing crops of plankton yet coral reef ecosystems have very high productivity. Photosynthetic production in reef communities is in the order of 5-20g carbon $\text{m}^{-2} \text{y}^{-1}$ (Sorokin, 1993) which is about two orders of magnitude greater than in the surrounding ambient waters (Lewis, 1977). This is largely because many benthic animals found on coral reefs live in mutualistic associations with symbiotic algae. While hermatypic corals are the most prevalent association, giant clams, flatworms, sponges, nudibranchs and sea anemones also benefit from symbiosis. These symbiotic algae can be present in high density within their host; in corals the algae are present at a density of 10^6 cm^{-2} of the corals' surface (Edmonds and Davis, 1986) and can account for 1-10% of the total annual global production of carbon by benthic organisms.

1.2 SYMBIOSIS AND ENDOSYMBIOSIS

DeBary defined the term symbiosis as "an intimacy of interaction between two organisms" in 1879. In current usage, symbiosis implies specificity and permanency rather than describing associations, which although beneficial to both partners, may be either accidental or transient. Endosymbiosis is an extremely close relationship and describes an association where one partner of the symbiosis is living within the other. This can be either intracellular, extracellular, or within host cavities. The larger partner in this situation is defined as the host.

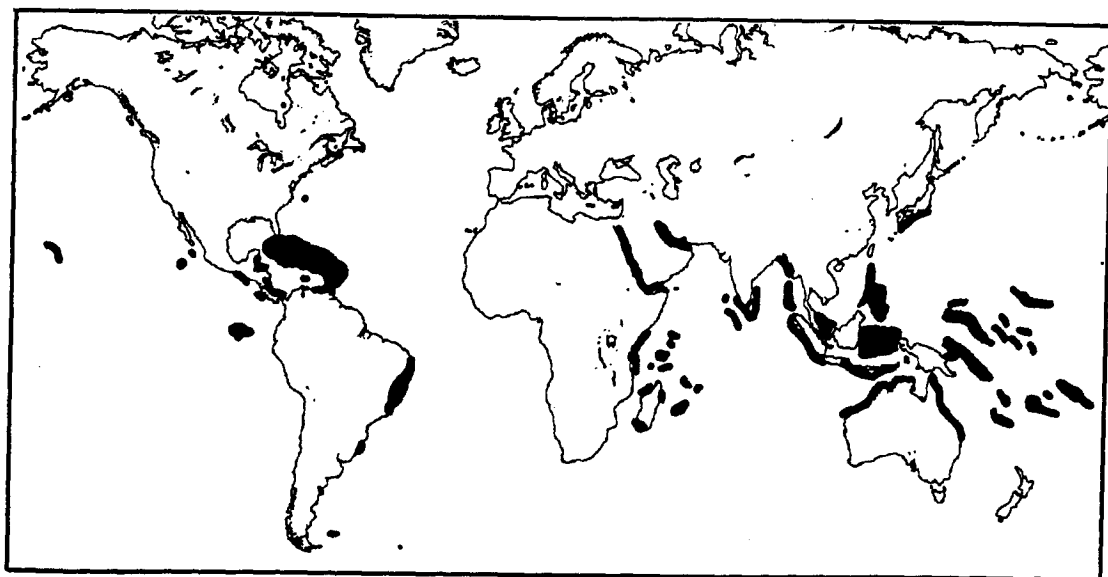


Fig 1.1 The present location of the world's coral reefs (shown in grey)

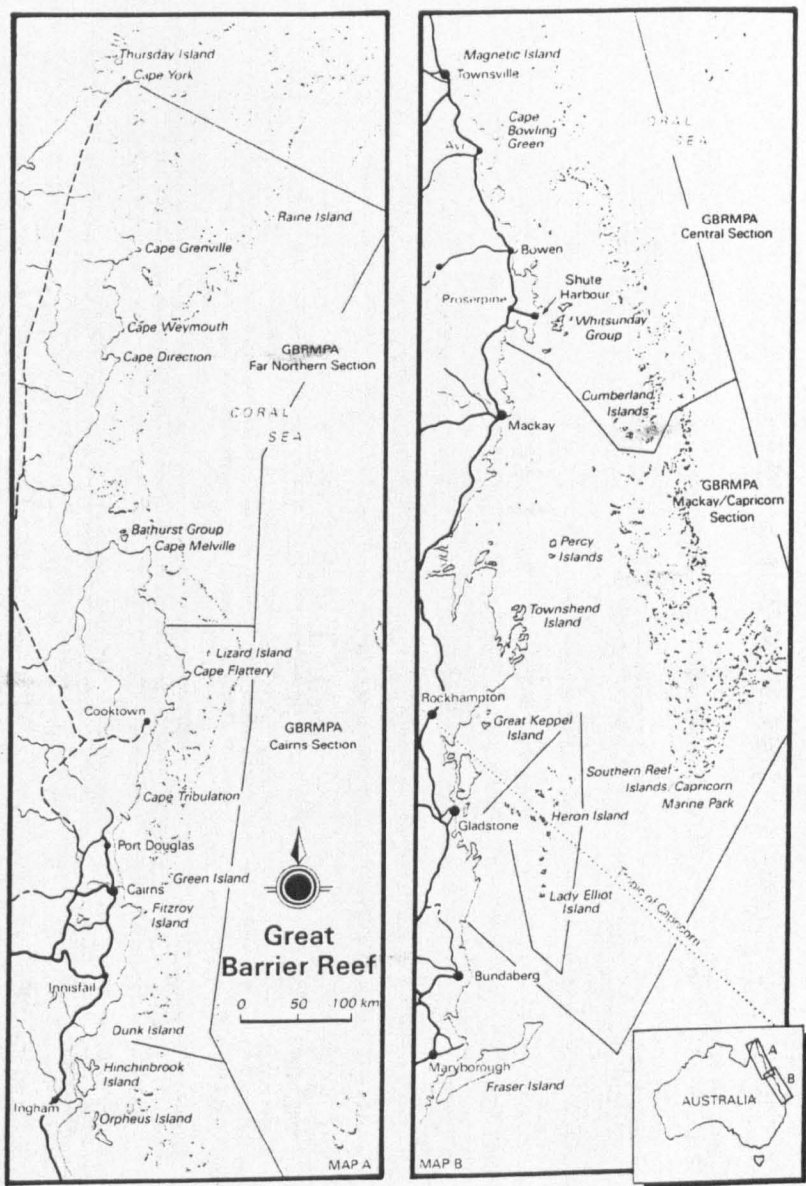


Fig. 1.2 Location of the Great Barrier Reef, Australia
(sourced from the Coral Reefs Lonely Planet Guide)

Algal/invertebrate endosymbioses are common in the marine environment and appear to be nutritional in nature. They occur in several marine phyla, Foraminifera, Cnidaria, Porifera, Tunicata, Platyhelminthes and Mollusca being the most common. Although aposymbiotic forms can be artificially produced in the laboratory, aposymbionts are rarely found in nature, presumably because the symbiosis confers evolutionary 'advantage' and is preferentially selected. Associations of corals (Coelenterata) and giant clams (Mollusca), with their endosymbionts, are often referred to as mutualistic (Allee *et al.*, 1949), although it is unclear whether the symbiosis is indeed beneficial to both partners. There is little evidence to suggest that the endosymbionts benefit from the partnership (Douglas and Smith, 1989). Indeed, several scientists have suggested that the hosts may be exploiting the endosymbionts, the host having a more parasitic role (Cooke, 1975). There are two main forms of endosymbiosis important to coral reef ecosystems, intracellular and extracellular. Within the giant clam the extracellular symbiotic algae are housed within small tubes radiating from the stomach diverticulum, the algae reaching this position during normal filter feeding activities (Norton *et al.*, 1992). In contrast, coral algal symbionts are located intracellularly generally within the gastrodermis, a tissue that lies very close to the animal's external surface. Symbionts reach this position by being phagocytosed into individual vacuoles (Trench, 1980).

Early research on the origin of endosymbiosis by Goetsch (1924) and Goetsch and Scheuring (1927) suggested a parasitic infection precipitated the initial symbiotic arrangement, only later to be followed by evolutionary modifications in the host. Goetsch (1924) experimentally infected *Hydra attenuata*, and found that infection was only achieved by compromising the animal's health by starvation and exposure to high temperature. Once infected the animal's health recovered and permanent associations resulted. Results of such experiments lead us to hypothesize that the oligotrophic environment, in which so many algal/invertebrate associations live, may well have been the driving force for the evolution of endosymbiosis.

More recent research has suggested that initial infestations were unlikely to be immediately successful in terms of a symbiosis. Cowen (1982) suggested that the symbiosis would be rather

like an arms race, both bionts trying to gain maximum benefit from each other whilst providing little in return. The symbiosis could then only become established once the algae placated the host, and consequently stopped its normal rejection mechanisms, by providing some beneficial factor. Other theories have been based on the properties of the bionts such as the animal habit of phagotrophy, algal motility such as in dinoflagellates, chemotaxis of symbionts to their hosts, the inability of carnivorous hosts to digest plant material and the algal resistance to digestion (Taylor, 1973a; Margulis, 1993).

Why so few animals form endosymbiotic relationships has been discussed by Smith (1991). Although endosymbiosis may have been important in the early evolution of some major eukaryote groups in the Precambrian (Margulis, 1993), since then endosymbiosis has contributed to the success of only a small number of phyla (Smith, 1991). All of these phyla have been aquatic with the large majority being lower invertebrates (Smith, 1991). The majority of the phyla are also simple in grade such as forams, sponges, flatworms and cnidarians, with thin tissue layers and relatively large surface area to volume ratios. All of these features may have predisposed them to invasion by algal symbionts. Major exceptions to this are the few bivalve molluscs involved in symbiotic relationships, *Tridacna*, *Hippopus*, *Corculum* and *Fragum*, all of which are highly complex.

Cyanophyta, Chlorophyta and Dinophyta are the main phyla of algae which form symbioses with invertebrates and are often referred to as Cyanellae (Pascher, 1929), Zoochlorellae and Zooxanthellae (Brandt, 1883) respectively, according to whether they are blue-green, green or yellow-brown in colour. Blue-green algae are common as marine endosymbionts in both sponges and ascidians, whereas green algae symbionts are common in fresh water; exceptions to this are associations with flatworms, large foraminifera, radiolarians and a few cnidarians. The most abundant of all symbionts are the dinoflagellates, which occur in forams, flatworms, cnidarians and molluscs.

1.2.1 The endosymbionts of Tridacnid clams

Three species of dinoflagellates have been described as endosymbionts in marine invertebrates, two from the genus *Amphidinium* and the third *Symbiodinium microadriaticum*. The algal endosymbionts of all Tridacnid clams, from now on referred to as zooxanthellae, are of the species *Symbiodinium microadriaticum* (Freudenthal, 1962). In situ, the zooxanthellae lack flagella, although evidence of flagellar apparatus is sometimes observed (Schoenberg and Trench, 1980b). When zooxanthellae are isolated and cultured, however, they revert to flagellated forms (Taylor, 1973a). *S. microadriaticum* is present as the endosymbiont of reef building corals, many sea anemones, gorgonians, hydroids and jellyfishes (Freudenthal, 1962; Taylor 1969, 1974; Loeblich and Sherley, 1979). Distinct strains of *Symbiodinium microadriaticum* have been distinguished by biochemical and morphological criteria in conjunction with algal cell size and host infectivity (Schoenberg and Trench, 1980a, 1980b, 1980c; Blank and Trench, 1985). Tridacnid clams can establish symbioses with many different strains of *S. microadriaticum* and it has been proposed that the growth rate of juvenile clams may be variable depending on with which strain a host establishes a symbiosis. Fitt (1985) demonstrated that the growth rates of juvenile *H. hippopus* infected with different strains of *S. microadriaticum* were directly related to the zooxanthellar growth rates in the hosts, those zooxanthellae with higher growth rates promoted higher host growth rates in the juvenile clams.

Although early work led to the consensus that the zooxanthellae lived in the haemal sinuses or in blood amoebocytes (Yonge, 1936), in *Tridacna* the algae are always present extracellularly. However, Norton *et al.* (1992) demonstrated that the zooxanthellae in the mantle are located in a series of tubules, arising from the stomach diverticulum. This tubular system radiates throughout the mantle tissue (Plate 1). Zooxanthellae are not confined to these tubules. They are also present in the thin epithelial coverings of the adductor muscle, visceral mass and pericardium (but not in the adductor muscle itself), in the gills (but at low abundance) and are found in abundance in the digestive mass of the animal (Norton *et al.*, 1992). However, those zooxanthellae in the digestive mass are typically degenerate. The zooxanthellar tubes possess very thin epithelial cell walls separating the zooxanthellae from the clam's blood (haemolymph)

system (Norton *et al.*, 1992) and it is likely that biochemical changes associated with the zooxanthellae may well be represented by a change in haemolymph composition. Changes in clam haemolymph glucose levels have been shown to be directly related to irradiance levels during a twenty-four hour cycle (diel cycle) in the clam *T. gigas*, and appear to be related to the photosynthetic activity of the zooxanthellae (Rees *et al.*, 1993).

Within the Tridacnid clam the zooxanthellae respond to light and give photosynthesis versus light intensity curves similar to any other free-living algae (Fisher *et al.*, 1985). The light response is very much dependent on the size of the clam. Small clams tend to reach maximal photosynthetic rates at one quarter of maximum sunlight intensities in air, whereas larger clams never reach maximum photosynthetic rates, presumably because of extensive shading of many of their zooxanthellae by both clam tissue and other zooxanthellae (Fitt, 1988). In response to low light conditions, zooxanthellae numbers rise in the host and/or pigment concentrations in the zooxanthellae increase. This strategy maximises photosynthetic efficiency despite inadequate irradiance (Mingoa, 1988). How this strategy is implemented is unknown as zooxanthellae numbers in host tissues tend to be constant, the host does appear to keep the zooxanthellae population 'in-check'. As a 'pair' the partners within a symbiosis probably benefit more nutritionally than if they were alone, a syntrophic arrangement. Indeed, early studies of Tridacnid algal/invertebrate relationships went as far as to suggest that the host farmed and harvested the algae, digesting them intracellularly when required (Yonge, 1936 and 1944). This is now believed to be unlikely, as intact algae pass straight through the host digestive system unharmed, and evidence now exists which indicates the leakage (often referred to as translocation) either by diffusion or cell mediated transport, of photosynthetic products from the algae to their invertebrate hosts. This finding has led to the term photobiosis. The transfer of compounds from algae to host in symbiotic associations has been demonstrated in several symbiotic algal/invertebrate relationships (Table 1.1). Whereas other studies have suggested a more mutualistic arrangement where both partners benefit from the association (Muscatine and Porter, 1977; Szmant-Froelich and Pilson, 1977; Benson *et al.*, 1978; Muscatine and D'Elia,

1978; Muscatine and Pool, 1979; Muscatine 1980 and Muscatine *et al.*, 1981; Wafar *et al.*, 1986).

Symbiotic algae from various coelenterates can make use of excretory products (McLaughlin and Zahl, 1959; McLaughlin *et al.*, 1964). These algae can utilise inorganic salts, urea, uric acid, guanine, adenine or any of 12 amino acids, glycerophosphoric, cytidylic, adenylic or guanylic acids as sole nitrogen and phosphorus sources. Nitrogen and phosphorus concentrations are typically in the range of 0.03-0.3 μM and 0.002-0.02 μM respectively in tropical reef waters (Larkum, 1983). The main nitrogenous excretion product in invertebrates is ammonium and it has been estimated that tissue ammonium levels of 5-50 μM are likely in algal/invertebrate symbioses (Wilkerson and Muscatine, 1984; Crossland and Barnes, 1977); levels that are several orders of magnitude greater than reef water nitrogen levels. In their analysis of haemolymph from the giant clam *T. maxima*, Deane and O'Brien (1980) found phosphate levels that were three-fold richer than those in the seawater from which the clam was removed. Clearly, the algae must benefit from the association by having a greater access to more of these nutrients within the host than they would in the free-living state.

The removal of excretory products by symbiotic algae must also benefit the host by reducing the metabolic energy expended by the host in waste removal. Simkiss (1964a and 1964b) noted that the presence of phosphate could also inhibit carbonate deposition. He suggested that the removal of phosphate from host tissues by the endosymbionts might actually promote calcification. Hallock (1981), in her mathematical model of algal symbiosis, demonstrated that the energetic advantage of this recycling in a symbiosis could be astounding, especially in the oligotrophic environment. Hallock calculated that energy available for growth and respiration by a symbiotic host was 1-2 orders of magnitude larger than that available to a non-symbiotic filter feeder.

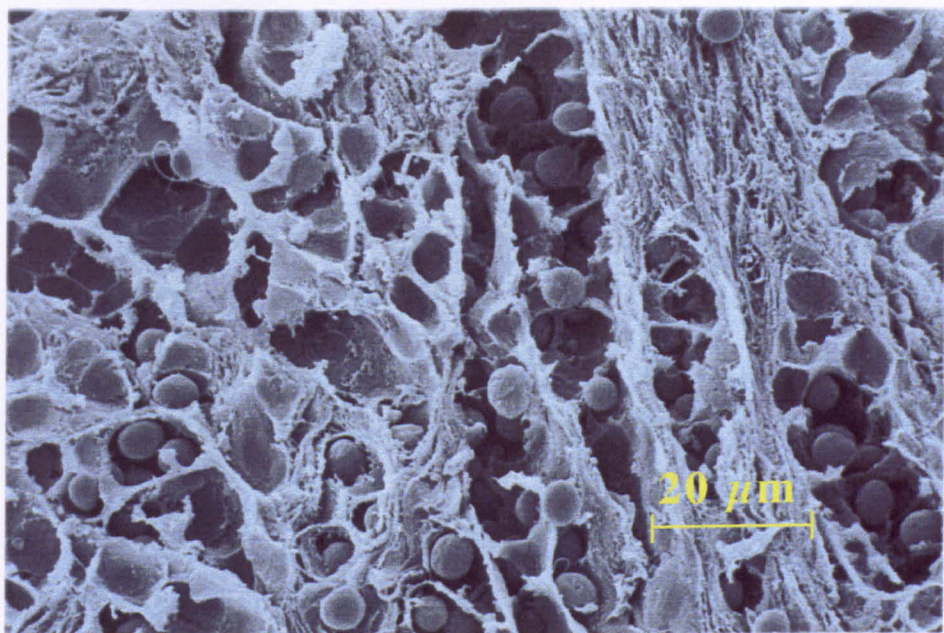


Plate 1. Zooxanthellar tubule system in *T. gigas*
(EM photograph supplied by Spencer Whitney and David Yellowlees)



Plate 2. *T. gigas*, maricultured animals at Pioneer Bay, Orpheus Island.
(Photographed by the author)

Table 1.1 Summary of possible translocated compounds in various algal/invertebrate symbioses.

<i>Host</i>	<i>Symbiont</i>	<i>Compound</i>	<i>Reference</i>
<i>Chlorohydra</i>	<i>Chlorella</i>	Maltose	Muscatine, 1965
<i>Hydra</i>	<i>Chlorella</i>	Maltose	Cernichiara et al., 1969
<i>Paramecium</i>	<i>Chlorella</i>	Maltose	Muscatine et al., 1967
<i>Spongilla</i>	<i>Chlorella</i>	Glucose	Muscatine et al., 1967
<i>Tridacna</i>	<i>Symbiodinium</i>	Glycerol	Muscatine, 1967
<i>Pocillopora</i>	<i>Symbiodinium</i>	Glycerol	Muscatine, 1967
<i>Cassiopeia</i>	<i>Symbiodinium</i>	Glycerol	Trench, 1971a and b
<i>Anthopleura</i>	<i>Symbiodinium</i>	Glycerol, alanine, glucose	Trench, 1971a and b
<i>Aiptasia</i>	<i>Symbiodinium</i>	Glycerol	Trench, 1971a and b
<i>Palythoa</i>	<i>Symbiodinium</i>	Glycerol	Trench, 1971a and b
<i>Chlorohydra</i>	<i>Chlorella</i>	Alanine	Lewis and Smith, 1971
<i>Convoluta</i>	<i>Platymonas</i>	Alanine, glycine	Muscatine, 1974
<i>Tridacna</i>	<i>Symbiodinium</i>	Glucose, oligosaccharides, glutamate, alanine, aspartate, serine, succinate	Streamer et al., 1988
<i>Acropora</i>	<i>Symbiodinium</i>	Hexose phosphates, Glucose, aspartate, malate, organic acids, succinate, glutamate	Streamer et al., 1993

In the majority of algal/invertebrate associations, the host retains the ability to feed as normal, with the translocated endosymbiotic products supplementing the host's diet rather than replacing it. Although the exact nature of released products in various symbioses has not yet been clarified, translocated photosynthates are generally thought to be simple molecules such as sugars, amino acids, lipids and perhaps organic acids, (Table 1.1). However, translocated molecules as in Table 1.1 do not provide a balanced diet and have been referred to as "junk

food" (Davies, 1984), the host having to maintain normal feeding to gain all the essential trace elements and nutrients required for growth. The translocated molecules are only thought to serve as an energy buffer in hard times or when energy intensive projects such as gametogenesis are undertaken, with the need for extra carbon. Several studies have suggested that the extra carbon may somehow be channelled into calcification in corals and giant clams (Goreau, 1959; Wainwright, 1963). If food is scarce or non-existent however, it is possible for a heterotrophic host to survive on the "junk food" provided by its symbionts. Fitt and Trench (1981) demonstrated that juvenile clams, containing their quota of endosymbionts, could grow in Millipore filtered seawater, with light as the only energy source, for over 10 months, with no detriment to the animals.

Smith (1991) considered the costs to the host in possessing symbionts. He suggested that the energetic costs of housing the algae, controlling their cell division, population size and exposing a large tissue surface area to maximise symbiont photosynthesis, might make photo-symbiosis less economic than the utilisation of photosynthetically fixed carbon in the form of phytoplankton and reduced vulnerability to predators. So the question arises why do the animals form such relationships if there is an obvious risk to housing the algae?

1.3 THE SYMBIOTIC RELATIONSHIP IN TRIDACNID CLAMS

Tridacnid clams (commonly known as the giant clams) are related to cockles and belong to the superfamily Cardiacea. There are eight species in the family, six of the species *Tridacna* (*T. gigas*, *T. derasa*, *T. crocea*, *T. maxima*, *T. squamosa*, and *T. tervora*) and two of the species *Hippopus* (*H. hippopus* and *H. porcellanus*). Six of these giant clam species exist in the Great Barrier Reef region (Figs 1.2, 1.3 and Plate 2).

All eight species possess endosymbionts. Aposymbiotic clams have never been reported, although occasional partially bleached clams do occur in otherwise normal populations. As noted by Fitt and Trench (1981) juveniles without their quota of zooxanthellae tend not to live beyond a few weeks. Members of the family Tridacnidae are protandrous hermaphrodites (Wada, 1954) with most juvenile clams remaining males until sexual maturity occurs, when the hermaphrodite

condition becomes apparent. Only four other marine bivalves are known to harbour algal endosymbionts, the bivalve *Corculum cardisa* (Linne) (Kawaguti, 1950), and the strawberry cockles, *Fragum fragum* Linnaeus, *Fragum unedo* Linnaeus (Kawaguti, 1983; Yamasu, 1988a and b) and *Fragum mundum* Reeve (Umeshita and Yamasu, 1985).

1.3.1 Distribution and habitat of the giant clams

The geographical distribution of Tridacnidae appears to have been influenced by the needs of their endosymbionts (Yonge, 1975). The clams are obliged to stay in areas with high and constant irradiance levels throughout the year and in shallow, low turbidity waters, to maximise available light for their photosymbiont partners, if the association is to be successful. They also require that the water has insufficient nutrients to sustain algal blooms which would also cut down on available light. *Tridacna* are thus restricted to the Indo-Pacific faunal region where all these requirements are normally met. The three largest species, *T. gigas*, *T. derasa* and *H. hippopus*, are limited to the western Pacific and Micronesia (Fig. 1.3). *T. crocea* has a slightly narrower range and that for *T. squamosa* extends from central Polynesia westward to east Africa. *T. maxima* is the most common of the Tridacnid clams and its distribution extends from East Africa to Southeastern Polynesia (Fig.1.3)

The large species of giant clams rely on their massive thickened shells to keep them in an upright position on the reef or sandy substrates, whereas smaller species attach themselves firmly to the substrate throughout their lives using a strong byssus. *T. crocea* and *T. maxima* also burrow deeply into coral so that only their mantle tissue extends from the surface.

1.3.2 Filter feeding in giant clams

Giant clams are the largest bivalves in the world. The species *T. gigas* is one of the largest bivalves ever known (Yonge, 1975), with the heaviest specimen on record, held in the American Museum of Natural History, New York (Miner, 1938), weighing in at 579.5 pounds (263 kilo). *T. gigas* can grow to over a metre in diameter, and it is difficult to believe that a bivalve can reach such sizes from filter feeding alone, since it is obvious that other filter feeding bivalves do

not. Stasek (1962) reported that the inhalent siphon in giant clams was very large and that it must permit the passage of extremely large volumes of water. He also noted that the gut contents of Tridacnids included large objects such as copepods and foram tests. This confirmed earlier experiments by Mansour (1946a and b) whom reported that copepods, ostracods, larvae of molluscs, eggs of fishes, echinoderms, protozoa, finer objects and endosymbiotic algae were often found in the stomachs of Tridacnid clams. Mansour concluded that, as the clams could utilise food particles up to 1.5mm in size, their immense size was due to their very efficient filter feeding system. The ability to process large volumes of water, and extract zooplankton as well as plankton from the water column, may indeed be beneficial in oligotrophic environments.

1.3.3 Lifespan and growth rates of giant clams

Early estimates of the lifespan of giant clams have varied from 8 years (Pelseneer, 1894) to several hundred years (Comfort, 1957) and the general consensus historically was that these bivalves had to be very old to have reached such immense size. The oldest reliably dated clam, is a large specimen of *T. gigas* dated, by counting the seasonal growth bands in the shell, at greater than 50 years old (Lucas, 1988).

It has been suggested that the Tridacnids are the fastest growing extant bivalves. It has been calculated that *T. gigas* grows at a rate sixteen times that of the common oyster (Bonham, 1965). Estimates of giant clam growth rates range from 2-12 cm/yr (Bartsch and Nichols, 1945; Rosewater, 1965 Beckvar, 1981; Heslinga and Watson, 1985; also references within *Giant Clams in Asia and the Pacific*; Sims and Howard, 1988; Price and Fagolimul, 1988; Nash, 1988; Crawford *et al.*, 1988a and b; Barker *et al.*, 1988; Solis *et al.*, 1988). Beckvar (1981) also noticed that not only did growth rates decrease with increasing shell length but also that the larger species generally had faster growth rates.

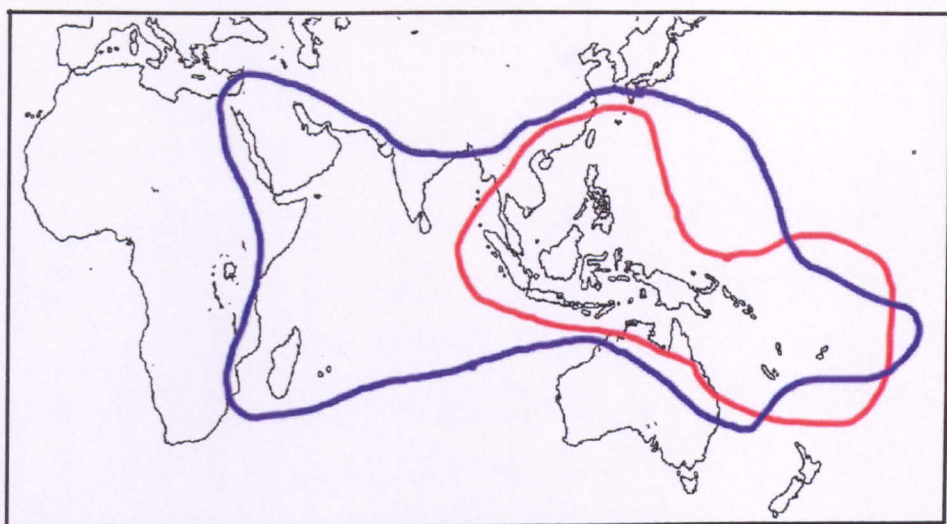


Figure 1.3 Geographical distribution of the species *T. gigas* (red line) and *T. maxima* (blue line). (Diagram after Yonge, 1975)

1.3.4 Maintenance of the symbiotic condition

For a symbiotic association to persist there must be an efficient way of passing on the symbiont from one generation to the next. The method of transmission varies in marine algal/invertebrate symbioses, either by dividing the symbionts between daughter cells at cell division (Foraminifera) or by infecting the eggs of an organism with the symbionts (Cnidaria). Often no obvious transmission method exists, in which case each succeeding generation needs to be reinfected by symbiotic algae to maintain the relationship. Tridacnid clam eggs are free of zooxanthellae (Mansour, 1946a; Norton and Jones, 1992), so the symbiosis in clams is established after metamorphosis (Fitt *et al.*, 1984; LaBarbara, 1975; Jameson, 1976; Fitt and Trench, 1981). Clam veliger larvae have to acquire all their zooxanthellae complement from the surrounding water, in the form of free living flagellated or coccoid algae during normal filter feeding activities. Newly metamorphosed clams have fewer symbionts. If starved of particles, and consequently the free-living symbionts, juvenile clams will not survive beyond metamorphosis (Southgate, 1988; Fitt and Trench, 1981). Motile zooxanthellae are apparently attracted to the host by the excretion of dissolved nitrogenous compounds such as ammonium or certain amino acids (Fitt, *et al.*, 1984; Fitt, 1985). Once the clam reaches the juvenile stage in its life history, the symbiotic association is fully established. Fitt *et al.* (1984) demonstrated that several different strains of *S. microadriaticum* could establish symbioses with *T. gigas*.

Zooxanthellae often remain in the stomach of veliger clams for several days, thus they are not digested by the host (Fitt *et al.*, 1986). Healthy zooxanthellae have been found in fecal pellets of several symbiotic hosts (Mansour, 1946a and b), including motile forms (Ricard and Salvat, 1977; Kempf, 1984) and subsequently cultured (Trench *et al.*, 1981). Non-digested zooxanthellae comprise up to 80% of the total mass of faecal pellets in *Tridacna* (Richard and Salvat, 1977). The establishment of symbiosis in the giant clam facilitates increased growth after metamorphosis, compared to animals devoid of symbionts (Fitt *et al.*, 1986). Once the association is established the algae proliferate but are checked as soon as they reach a population

density appropriate for the environment that they are in. The host clam maintains the zooxanthellae numbers (by an unknown mechanism), a dynamic stability characteristic of this symbiosis. In a general way, this control may be achieved simply by what is supplied to the algae in the form of catabolic or respiratory products.

1.3.5 Structural modification in giant clams

Once established, a symbiosis often manifests in some modification in each partner, either biochemically or structural. Evolutionary selection for such modifications, tend to make the association more efficient and increase the dependency of one biont on the other. The shell and mantle of the Tridacnid clams, in particular, have a structural modification, which increases the sunlight available for symbiont photosynthesis. The shell orientation has altered so that the mantle tissue faces in an upward direction. The mantle has also become greatly enlarged so the mantle spills over the shell margin. The shell structural adaptation does not appear to affect its normal capacity as a filter feeding bivalve as giant clams are efficient filter feeders (Mansour, 1946b). In addition the mantle of the clam contains pigments which may serve to protect the zooxanthellae from excessive light intensities; they may also affect the quality of light that reaches the zooxanthellae and indirectly alter the character of photosynthate produced by the algae (Taylor, 1973b).

1.4 TRANSLOCATION OF ALGAL PHOTOSYNTHATES

The first indications that some invertebrates were partly autotrophic were the observations that they produced excess oxygen when exposed to light (Yonge, 1932; Gohar, 1940) and that starved symbiotic animals exhibited reduced weight loss compared to aposymbionts (Muscatine *et al.*, 1967). Giant clams in particular, were also noted to be much larger than non-symbiotic contemporaries. The generally accepted interpretation for these observations was that the zooxanthellae have an important nutritional role, providing nutrients to prevent the loss of host tissue reserves. The main questions, that remain unanswered, were how much material was translocated to the host, and what was actually provided by the algae.

Since the introduction of the ^{14}C -tracer technique to measure primary productivity in aquatic systems, it has been used extensively to trace the products of photosynthesis in symbiotic associations. The technique involves the incubation of the animal in radioactively labelled carbon substrate (such as sodium bicarbonate) and subsequent measurement of the radioactivity partitioned between the algal cells and medium, in *in vitro* studies, and between algal cells and host tissues, in intact animals. The translocated compounds can then be identified by classic chromatography techniques. Initial evidence for the photosynthetic contribution of algae to the host's nutrition in a symbiotic relationship came in 1958, when Muscatine and Hand demonstrated that ^{14}C carbon dioxide tracer partitioned from the symbiotic algae into host tissues in the sea anemone *Anthopleura elegantissima*. At that time, however, there was no indication to the type of compounds that were being translocated to the host, or any indication of how those compounds may be utilised. To date radiotracer techniques have been one of the major methods of determining nutrient pathways in symbiotic relationships (Smith *et al.*, 1969; Muscatine *et al.*, 1972; Muscatine, 1973; Taylor, 1973b). There are however several problems with the use of the ^{14}C -radiotracer technique. ^{14}C : ^{12}C disequilibria in short-term experiments and re-fixation of ^{14}C in longer experiments can cause significant under estimations of the translocation, also normal filter feeding carbon sources can interfere (Muscatine *et al.*, 1984). It is often difficult to isolate the zooxanthellae from all the host tissues, soluble products and lysis products may also be washed away in the zooxanthellae isolation, or catabolized to carbon dioxide during the incubation. In view of these problems, scientists sought alternative methods of estimating translocation quantitatively using symbiont growth rates.

Muscatine *et al.*, (1983,1984) devised a method for determining total daily translocation, which did not utilise ^{14}C experiments. Based on the hypothesis that, of the total net carbon fixed per day only a fraction is used in the growth of the zooxanthellae which leaves the remainder of fixed carbon to be translocated to the host. It is assumed that the rate of photosynthesis in zooxanthellae is similar to that of free living algae.

$$T = ((\mu_c - \mu) + \mu_c) \times 100$$

Where:

T = the estimated daily translocation as a percentage of the total net carbon fixed.

μ = the specific growth rate of the zooxanthellae, which represents the daily increase in algal standing crop, usually determined by algal cytokinesis.

μ_c = the carbon specific growth rate, estimated from the standing stock of algae, cell carbon and the net production rate of the algae measured as the quantity of carbon acquired each day.

In their study of the anemone *Condylactis gigantea*, Battey and Patton (1987) demonstrated that all the translocated substrates in this association were respired. In the advent of the growth rate method for estimating algal translocation, the assumption that all the translocated carbon is respired, an earlier model proposed by Muscatine *et al.* (1981) has been employed to calculate the contribution of zooxanthellae-translocated carbon to host respiration (CZAR) in several algal/invertebrate symbioses. Results from such models for juvenile Tridacnid clams indicated that 7-137% of a host's metabolic carbon demand could be met by translocated zooxanthellar fixed carbon, the range reflecting variable photosynthesis and respiration rates (Fitt *et al.*, 1986). Numerous experiments have been carried out on isolated zooxanthellae to attempt to trace nutritional pathways in the various symbiotic associations, however much has still to be done on the intact Tridacnid/algal relationship. To date there remains controversy as to the exact nature of translocated material and whether lipids in some simple form such as glycerol or fatty acids are actually translocated to the host clam. Experiments carried out on *Symbiodinium microadriaticum* often isolated from coelenterate hosts have been used to describe the likely translocation products in all symbiotic associations hosting that particular symbiont. In fact,

there are good reasons to doubt this assumption. Glycerol for instance was found to account for as much as 95% of the total ^{14}C recovered in *in vitro* studies of isolated zooxanthellae (Taylor, 1973b) yet isolated zooxanthellae typically do not behave in the same way when cultured as they do *in situ* (Smith *et al.*, 1969). Freshly isolated zooxanthellae do not release large amounts of photosynthate unless they are incubated with host tissue homogenates (Muscatine, 1967; Trench, 1971b; Muscatine *et al.*, 1972). Isolated zooxanthellae also tend to be fragile, especially those from coelenterates, and it is difficult to separate those products excreted, from those that may be released due to cell lysis. Initial studies implicated glycerol as the major translocated molecule in several symbioses, including giant clams, with other compounds such as glucose, aspartate, glutamate, succinate, alanine being translocated in smaller quantities (Muscatine, 1967; Muscatine and Cernichiria, 1969; Trench, 1971a+b; Schmitz and Kremer, 1977; Hoffman and Kremer, 1981). However, more recent studies suggest that glucose may be a significant translocated substance (Streamier *et al.*, 1993).

1.4.1 Evidence for translocation in the giant clam symbiosis

The earliest evidence for the actual translocation of photosymbiont products in giant clams was in 1965 when Goreau *et al.* demonstrated the partitioning of radiotracer into host tissues in the clam *T. elongata* (not a new species but an elongated form of *T. maxima*). Isolated zooxanthellae from *T. maxima* have been found to excrete glycerol, glucose and alanine in culture, however only in significant quantities when host tissue homogenate is added to the culture (Muscatine, 1967; Masuda, 1994). There are several reasons to doubt that glycerol is a major translocated product in the intact giant clam symbiosis. Glycerol is an unusual compound to be found excreted by algae, especially in the quantities seen in zooxanthellar cultures, algae normally excrete simple sugars and amino acids such as alanine and glutamic acid (Hellebust, 1965 and 1967). Experiments carried out by Streamier *et al.* (1988;) and Rees *et al.* (1993) have found glucose to be the major translocated compound in *Tridacna*. Rees *et al.* (1993) sampled haemolymph in *T. gigas* during a diel cycle and found appreciable glucose, concentrations of 400 μM were not uncommon during high irradiance, however glycerol remained negligible or

was absent. To date there remains conflicting evidence for the nature of translocated material in the giant clam symbioses.

1.5 LIGHT-ENHANCED CALCIFICATION

Coral reef ecosystems produce over half of the total carbonate material laid down in the world's oceans today. Gross carbonate production on coral reefs has been estimated at around 1-35 kg m⁻²yr⁻¹ (Chave *et al.*, 1972), although a range of 1-10 kg m⁻²yr⁻¹ appears to be more likely (Land 1979). Such reefs have existed for 450 million years (although the ancient corals were of different taxa to those of extant reefs), making them one of the oldest ecosystems on the planet. The early coral reefs covered much larger areas than those of today but were wiped out in the first wave of mass extinctions of marine life at the Permian-Triassic boundary, 225 million years ago (Veron, 1995). Early experiments on the calcification rates in reef building (hermatypic) corals, giant clams and calcareous algae (measured by radiotracer techniques) found photosynthesis by their endosymbionts resulted in increased rates of calcification by the host. This feature has led to the term light-enhanced calcification (Goreau, 1959; Goreau and Goreau, 1960; Goreau, 1977).

1.6 REEF POLLUTION

The development of agriculture and tourist industries near coastal areas in Australia has increased the introduction of terrestrial pollution into reefal waters, with artificial fertilisers, pesticides, detergents and waste water discharges finding their way into the reef ecosystem. It is thus imperative to evaluate the effect of raised nutrients on photo-symbiotic animals so that we can identify such stresses early on, to prevent further deterioration of these ecosystems. To address this problem part of this thesis examines the effect of elevated nutrients on skeletal carbonate isotopic signatures of giant clams. This work investigated the possibility of identifying eutrophication 'stress' in giant clams by micro-sampling shell carbonate and subsequent analysis of the trace element strontium and the stable isotopes carbon and oxygen.

Pollutants threaten the balance of barrier reefs, as the introduction of higher nutrient levels causes an increase in benthic and pelagic algae. The proliferation of macrophytic algae tends to suffocate the existing benthic species, especially those such as corals that have no mechanism for ridding themselves of the algal covering and grow more slowly than the opportunistic macrophytes (Rasmussen and Cuff, 1990). Algal blooms also occur with increased nitrates (Bell and Gabric, 1991), and cut down available light for symbiont photosynthesis, which in turn leads to increased sedimentation rates. Proliferating bacteria, growth enhanced by the excess nutrients and dying coral mucus, also utilise all the available oxygen in the water column causing anoxic conditions. Eutrophication also provides enhanced food supplies for boring sponges, which weaken coral structures (Rose and Risk, 1985). All these and other effects are thought to cause the destabilisation of reef corals and their associated species, for which the corals often provide mechanical support. Previous experiments on Tridacnid clams have demonstrated that increased nutrients cause a rapid increase in the organic biomass of the animal but normal skeletal carbonate deposition tends to be disrupted. The skeletons tend to be more fragile and less massive making them more prone to breakage (Belda *et al.*, 1993a).

1.7 STRONTIUM IN SHELL CARBONATE

This thesis also examines the strontium content in giant clam carbonate, in conjunction with stable isotope analysis, in-order to address the question of whether strontium could be used to identify pollution, in the form of eutrophication, events in these animals. The overwhelming bulk of a coral reef is calcium carbonate with magnesium and strontium providing the greatest proportion of co-precipitated cations. Odum (1957) demonstrated that the Sr/Ca ratio in invertebrate skeletons was directly proportional to the Sr/Ca of the ambient seawater. Sr/Ca ratios have since been shown to be accurate records of temperature and salinity in corals collected from oligotrophic waters (Beck *et al.*, 1992). Pollution by agricultural runoff however, is thought to blur the temperature signal because of chemical and morphological alteration of the coral aragonite. This feature thus initiated the analysis of strontium content as a possible proxy for anthropogenic reef disturbance (Rasmussen, 1988). Controversy exists as to whether

bivalve mollusc strontium contents are reliable proxies for seawater temperatures and/or pollution events or whether they merely reflect the growth rate of the animal (deVilliers *et al.*, 1994). Dodd (1965) for instance found conflicting results for *Mytilus sp.* with strontium concentrations being either negatively or positively correlated with temperature, dependent on which part of the shell he sampled.

1.8 STABLE ISOTOPES OF CARBON AND MASS SPECTROMETRY

^{14}C Carbon, used in the tracer experiments outlined previously, is a relatively short-lived radioactive nuclide whereas natural carbon essentially consists of a mixture of two stable isotopes, ^{12}C (98.9%) and ^{13}C (1.1%) with a corresponding $^{12}\text{C}/^{13}\text{C}$ ratio of 89.42 (Schidlowski, 1986). The advent of the gas capillary mass spectrometer in the late 1930's provided the scientist with a machine that could measure the stable isotopes of carbon. Pioneering work by Nier and colleagues demonstrated that reduced biological carbon compounds tended to be enriched in the lighter isotope, whilst inorganic carbon such as carbonates are ^{13}C -enriched (Nier and Gulbransen, 1939; Nier, 1947). As isotopes are unevenly distributed in natural organic and inorganic compounds it was realised that this feature could be used to reveal information about the processes involved in the formation of particular compounds.

Conventional analyses of the isotopes of carbon involve the comparison of carbon dioxide, produced from the combustion of an organic sample or from the dissolution of a carbonate, to a carbon dioxide standard of known isotopic composition. The standard gas, either produced in-house or purchased, is calibrated against an internationally recognised standard. Isotopic ratios, corrected for any instrumental effects and for the presence of a minor isotope of oxygen ^{17}O , are converted to a $^{13}\text{C}/^{12}\text{C}$ abundance ratio or in the case of oxygen a $^{18}\text{O}/^{16}\text{O}$ ratio. The ratios are more commonly expressed as $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values, in units per mil (‰), relative to the Pee Dee belemnite (PDB) from the Pee Dee formation of Carolina which was assigned a $\delta^{13}\text{C}$ value of 0‰ (Urey *et al.* 1951, Craig 1957, Chisholm *et al.* 1982), as in the equation below;

$$\delta^{13}\text{C (or } \delta^{18}\text{O)} \text{ ‰} = ((R_{\text{sample}} - R_{\text{standard}}) \div R_{\text{standard}}) \times 1000$$

Where $R = {}^{13}\text{C}/{}^{12}\text{C}$ or ${}^{18}\text{O}/{}^{16}\text{O}$

Positive values of $\delta^{13}\text{C}$ (or $\delta^{18}\text{O}$) indicate an enrichment of ${}^{13}\text{C}$ (${}^{18}\text{O}$) relative to PDB whereas negative values indicate depletion. The supply of the Peedee belemnite has long been exhausted so it is no longer possible to calibrate mass spectrometers directly to this standard. Recent guidelines have suggested that carbon and oxygen isotopic data should be expressed in per mil relative to VPDB by assigning a value of +1.95‰ and -2.2‰ exactly for the NBS 19 calcite standard (Coplen, 1996). In this study, the use of PDB has been retained as there is at present no internationally recognised organic compound standard for continuous flow isotope ratio monitoring gas chromatography mass spectrometers (IRM-GCMS). Where PDB values are quoted in the clam carbonates section, four analyses of NBS19 are also included for reference, although raw data were actually normalised to an in-house carbonate SPAR standard of which numerous analyses were available.

1.8.1 Fractionation of isotopes - kinetic effects

The fractionation of isotopes observed in biological systems primarily occurs because of two mechanisms: kinetic fractionation and thermodynamic fractionation; the distinction being between non-equilibrium and equilibrium situations. In kinetically controlled systems, such as the enzymatic reactions of living cells, the isotopic composition of the reaction products can vary markedly from the initial substrate. Kinetic isotope effects originate in the nature of vibrational energy levels of chemical bonds. Bonds involving heavier isotopes have a higher potential energy than any bonds involving lighter isotopes. A ${}^{13}\text{C}$ - ${}^{13}\text{C}$ bond is stronger than that of ${}^{13}\text{C}$ - ${}^{12}\text{C}$ for instance. This effect can lead to differences in reaction rates when a change in bonding at the carbon position occurs, since a bond involving a lighter isotope is potentially 'easier' to break. Kinetic isotope effects are usually greater than unity, as the heavier isotope bond is the stronger, and can be predicted using the relationship; $k_x + k_y \neq 1$, where k_x is the reaction rate for the lighter isotope and k_y for the heavier isotope. Although there are several possible isotope

effects the most significant are primary isotope effects whereby a bond containing an atom in consideration is changed (broken) in the rate-determining step. Kinetic isotope effects thus result in reaction products that are isotopically dissimilar to the substrate in the reaction when reactants are provided in excess. In carbon, kinetic isotope effects for ^{13}C versus ^{12}C are very small and usually in the range of 1.01 to 1.10, resulting in ^{13}C -depleted products compared to the substrate by 1 to 10‰. It is important to note at this point however, that if no carbon-containing bond is involved in a particular reaction then there is unlikely to be a primary isotope effect (Fig. 1.4).

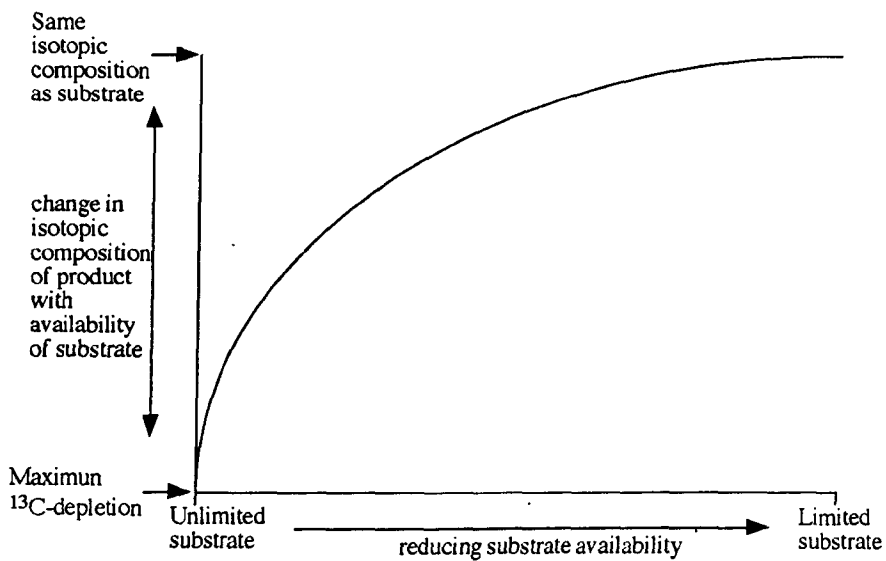
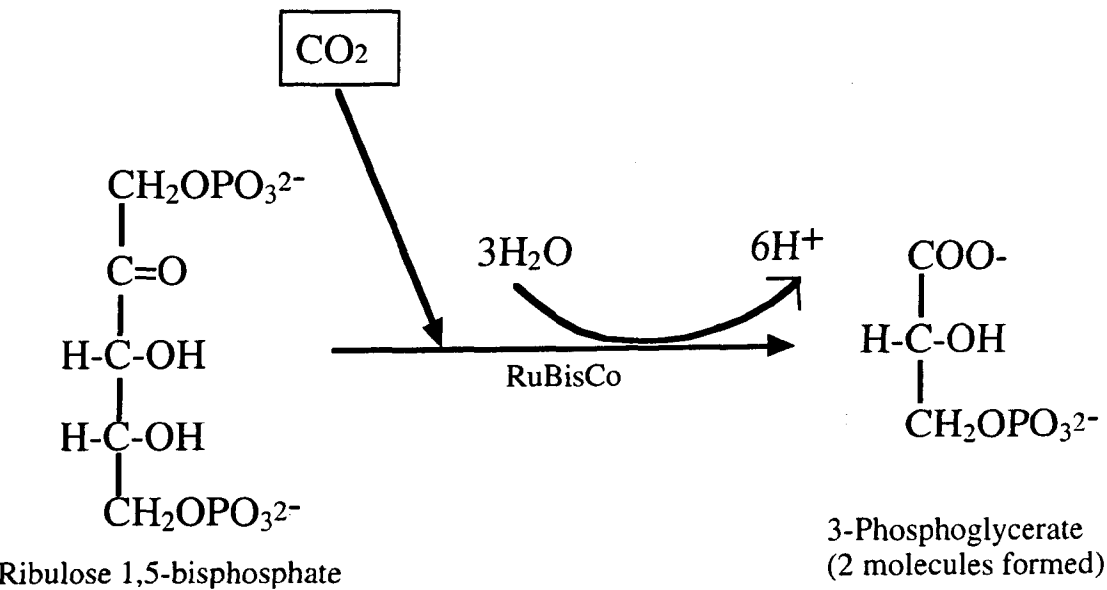


Fig 1.4 Diagram to illustrate a change in isotopic composition of a product with availability of substrate, in an enzymatic reaction in which the enzyme discriminates against the isotope ^{13}C , such as in RuBisCo.

Enzymes typically discriminate against the heavier isotope of carbon. Carbon isotope discriminations in enzymatic reactions are commonly in the range of 0-20‰, however values as large as 60‰ are occasionally observed (O’Leary, 1981). Isotope discriminations are nearly always fully exhibited when an excess of substrate is available, however if the substrate is limited, isotopic fractionation is lessened, as substrate is used up the enzyme is increasingly obliged to use isotopically heavier substrate. In the case where all the substrate is consumed, and is not replenished, the product will have the same isotopic composition as the original substrate $\delta^{13}\text{C}$.

The enzyme responsible for the fixation of carbon in C3 plants, Ribulose-1,5-bisphosphate carboxylase/oxygenase, commonly referred to as RuBisCo, discriminates against ^{13}C (Estep et al 1978; Wong et al 1979) (Fig. 1.5)

Fig 1.5. Overview of C3 photosynthesis



The products of C3 photosynthesis, therefore, are typically ^{13}C -depleted compared to the original carbon dioxide taken up by the plant (atmospheric CO₂ = -7‰) and have $\delta^{13}\text{C}$ values in

the range of -25 to -35‰ (Wong and *et al.*, 1979; O'Leary 1981). In contrast, the $\delta^{13}\text{C}$ values of C4 plants are typically in the range of -11.9 to -15.2‰ (Troughton *et al.*, 1974; Bender, 1968; Bender, 1971; Smith and Epstein, 1971). The reason for this isotopic difference mainly lies in the mechanisms employed for carbon fixation. The enzyme RuBisCo fixes carbon dioxide into a 3-carbon compound, 3-phosphoglycerate, which is subsequently fed into the Calvin cycle. The isotopic fractionation of photosynthetic products from the original fixed carbon dioxide, seen in C3 plants, is primarily due to isotopic discrimination of this enzyme.

The initial carbon fixation of C4 plants involves the enzyme phosphoenolpyruvate (PEP) carboxylase and produces a 4-carbon intermediate, oxaloacetate. The isotopic fractionation of PEP carboxylase is comparatively small, at around 0.5-3.6‰ (Whelan and Sackett, 1973; Reibach and Benedict, 1977; O'Leary, 1981). Oxaloacetate is converted to either aspartate or malate, which is transported from the mesophyll cells to the bundle sheath cells, where malate and/or aspartate are decarboxylated and the carbon dioxide released is refixed by RuBisCo. This mechanism provides distinct packets of carbon dioxide and RuBisCo enzymatic isotopic discrimination cannot be fully exhibited because of limited carbon supply.

A third class of plants, crassulacean acid metabolism (CAM) plants, have $\delta^{13}\text{C}$ values that overlap those values for C3 and C4 plants and range from -11 to -27‰. CAM plants are able to fix CO₂ in the light (using RuBisCo) or in the dark. Dark fixation involves the carboxylation of phosphoenolpyruvate and its subsequent reduction to malate. The malate accumulates in the plant vacuole where it remains until the next 'light' period when it is decarboxylated and the released carbon dioxide is fixed by RuBisCo, again in distinct packets. The range in $\delta^{13}\text{C}$ values is thus an indication of the predominant fixation mechanism at the time, more ¹³C-enriched values indicating 'dark' fixation, and those more ¹³C-depleted values, similar to typical C3 values, indicating fixation in the 'light' (Bender *et al.*, 1973). Although the isotopic discrimination of RuBisCo primarily dictates the isotopic fractionation of carbon during carboxylation, there are other factors which affect the final isotopic composition of the

photosynthetic products, the diffusion of carbon dioxide into the plant before fixation, for instance, is also associated with a kinetic isotopic fractionation.

Reports of $\delta^{13}\text{C}$ values of animals from marine, freshwater and terrestrial environments are very similar to the $\delta^{13}\text{C}$ values of the plants taken from the same environment (Craig, 1953; Degens, 1968; Degens *et al.*, 1968; Sackett *et al.*, 1965; Smith and Epstein, 1970). This led to the discovery that there was only a small isotopic difference between an animal's diet $\delta^{13}\text{C}$ values and their whole body tissue $\delta^{13}\text{C}$ values, typically only 1-2‰ or less (DeNiro, 1977). Herbivores consuming only C4 plants could be distinguished from those consuming only C3 plants, from analysis of their bulk $\delta^{13}\text{C}$ values, animal tissue $\delta^{13}\text{C}$ values in between typical C3 and C4 compositions suggesting a mixed diet. At each trophic position in a food web the organism is also ^{13}C -enriched relative to its diet (DeNiro and Epstein, 1978) and this observation meant that dietary links could be deduced from food-chain samples from the analysis of natural carbon isotopes. Although metabolic processes can change the initial $\delta^{13}\text{C}$ signature of the food consumed, generally metabolic effects are small. $\delta^{13}\text{C}$ variations of greater than 6‰ between food sources and whole animal tissues are therefore interpreted as the utilisation of foods of different isotopic composition rather than due to individual animal metabolism (Mosara *et al.*, 71, Minson *et al.*, 75 and DeNiro and Epstein, 78). Numerous studies have been carried out on terrestrial (Fry *et al.* 1978; Tiesen *et al.*, 1979), freshwater (Rau, 1978 and 1980) and estuarine (Fry and Parker, 1979; Haines and Montague, 1979 and Stephenson and Lyon, 1982) ecosystems in view of tracing carbon through the systems using bulk tissue $\delta^{13}\text{C}$ analysis.

Stable isotope ratios have been extensively used to estimate diet composition in marine food chains (Parker, 1963; McConnaughey and McRoy, 1979; Rau and Hedges, 1979 and Rau *et al.*, 1983), although many reports on marine food webs have often been surveys of isotope ratios rather than investigations of metabolic pathways. Carbon isotopic compositions of aquatic plants

range between -11 and -39‰ (Farquhar *et al.*, 1989) which has led to the belief that some may be utilising C4 photosynthetic pathways, however C4 plants are generally not found in the aquatic ecosystem. In particular the physiological adaptation, in the form of bundle sheath cells, required for the concentration of carbon, does not exist in aquatic plants. It is more likely that the small isotopic fractionations observed are either due to the slow diffusion of carbon dioxide in water across boundary layers, or in the case of marine algae, the use of bicarbonate in addition to carbon dioxide, from the water column. In conditions where slow diffusion dominates the supply of carbon dioxide, the supply of carbon becomes limiting and RuBisCo isotopic discrimination is not fully exhibited, the enzyme simply cannot be selective when there is finite substrate. If all reactants are consumed and converted to product in an irreversible reaction, there is no fractionation (see Fig. 1.4). The isotopic composition of bicarbonate in seawater is some 7‰ ¹³C-enriched compared to the isotopic composition of dissolved carbon dioxide. Therefore, the direct utilisation of bicarbonate by algae is a mechanism by which would result in ¹³C-enriched photosynthetic products and subsequent whole algal cells, ¹³C-enriched compared to that obtained from the utilisation of dissolved carbon dioxide solely. However, it should be noted that the preferred carbon species utilised by RuBisCo is carbon dioxide and that the enzyme carbonic anhydrase, typically associated with the algal cell walls, catalyses the conversion of bicarbonate to carbon dioxide at the site of carbon fixation in such bicarbonate utilisation by algae. To date it is unknown whether this enzymically mediated conversion possesses an isotopic discrimination. These points are particularly important in the context of the study of the carbon supply to zooxanthellae, within giant clam tissues, and are discussed further in Chapter 3. As aquatic plants yield isotopically ¹³C-enriched values compared to many terrestrial plants this difference has been used to distinguish between aquatic and terrestrial diets of ancient populations and more recently in the dietary study of arctic foxes (Chisholm *et al.*, 1982; Gilmour *et al.*, 1995).

1.8.2 Fractionation of isotopes - thermodynamic effects

Thermodynamic fractionations are isotope fractionations occurring for processes which are at equilibrium and are often referred to as 'equilibrium effects' in older literature. Thermodynamic effects represent the balance of two kinetic effects at chemical equilibrium and are therefore usually smaller than individual kinetic effects (Farquhar *et al.*, 1989). Thermodynamic effects, like kinetic effects, are temperature dependent. Of particular interest to this study is the biological fractionation of carbon and oxygen isotopes in biological carbonate. This effect is exhibited in the dissociation and subsequent equilibration of carbonate in water to produce bicarbonate and carbon dioxide and is important for the study of shell carbonate of giant clams and other calcifying aquatic organisms. The carbonate system can be represented by a series of equations. An isotope effect is associated with each of these equilibria, the isotopic compositions of precipitated carbonate and dissolved carbon dioxide are thus different (Table 1.2).

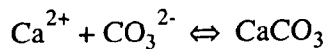
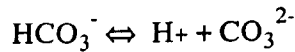
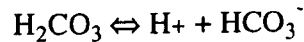
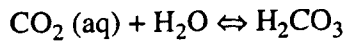


Table 1.2 Oxygen isotope fractionation factors with respect to water for the system $\text{CO}_2\text{-H}_2\text{O}$.

	$10^3 \ln a_{\text{H}_2\text{O}}$	$DG^\circ (\text{cal/mol})$	Source
H_2CO_3 (25°C)	38.8	-22.7	McCrea (1950)
H_2CO_3 (19°C)	38.7	-22.5	Usdowski et al. (1991)
HCO_3^- (25°C)	34.0	-20.2	McCrea, (1950)
HCO_3^- (19°C)	34.5	-20.0	Usdowski et al. (1991)
CO_3^{2-} (25°C)	18.1	-10.7	McCrea, (1950)
CO_3^{2-} (19°C)	18.2	-10.6	Usdowski et al. (1991)
CO_2 (gaseous) (19°C)	41.6	-24.1	O'Neil and Adami (1969)
CO_2 (aqueous) (19°C)	56.3	-32.7	Usdowski et al. (1991)

Calcium carbonate precipitated from water by marine organisms has an oxygen isotopic composition ($\delta^{18}\text{O}$) which is temperature and salinity dependent (Urey, 1947; McCrea, 1950; Epstein *et al.*, 1951; Epstein *et al.*, 1953). Provided the original isotopic composition of the water is known (or can be estimated) and an animal deposits its carbonate in equilibrium with the surrounding water, biogenic carbonates can be used to infer water temperatures. This feature has been used extensively to infer palaeotemperatures in fossil corals, molluscs and foraminifera. The isotopic compositions of foraminifera, which were isolated from deep-sea sediments, has provided information as to the climatic variations of the last million years (Emiliani, 1966; Shackleton, 1967). Not all calcifying organisms precipitate their carbonate in isotopic equilibrium, corals in particular appear not to precipitate their carbonate in isotopic equilibrium with the surrounding water (Erez 1978; Swart, 1983 and references therein). This fact has led to the belief that there is a so-called 'vital' effect, which causes these disequilibria, and the presence of zooxanthellae within corals has been one of the factors postulated for the effect. The aspect of carbonate stable isotopic disequilibrium is dealt with in more detail in Chapter 5.

The factors controlling the $\delta^{13}\text{C}$ value of biologically deposited calcium carbonate are complex. The temperature dependent isotopic fractionation of carbon is small (0.035‰ per °C) and it is thought that the $\delta^{13}\text{C}$ of skeletal carbonate (calcitic or aragonic) is more likely to mirror the ambient dissolved inorganic carbon in the water (Mook and Vogel, 1968, Fritz and Poplawski, 1974). Changes in isotopic composition of biogenic carbonate can thus occur with changing isotopic composition of the water mass or from physiological processes. The ΣCO_2 in water has a $\delta^{13}\text{C}$ which has a diurnal cycle, $\delta^{13}\text{C}$ values in the day are higher than the mean for 24 hours (Weil *et al.*, 1981), high photosynthetic activity by algae, during high irradiance, preferentially removes ^{12}C from the water column and leaves ^{13}C -enriched water behind. The process of skeletal calcification may also source the carbon from so called 'metabolic carbon dioxide', as well as from the water column, and thus exhibit $\delta^{13}\text{C}$ values which are more ^{13}C -depleted than

that of carbonate precipitated at equilibrium (Jones *et al.*, 1986; Romanek and Grossman 1989 and Romanek *et al.*, 1987).

In the study of biological tissues, it is the kinetic effects of enzymatic isotopic fractionations that are generally considered, because of their relative magnitude compared to that of thermodynamic effects. However it is necessary to note that there may be associated thermodynamic effects in these reactions, when not enzyme mediated, whose isotopic fractionations are not taken into account (O'Leary, 1981).

1.8.3 Isotopic composition of biochemical fractions

All cells contain a suite of different classes of compounds. Lipids, sugars and proteins are the most predominant and have defined cellular roles. Lipids are involved in membrane structure and used as energy storage compounds, sugars are used as energy supplies and proteins have important structural functions. The analysis of a whole body or indeed a particular tissue in the body gives an average $\delta^{13}\text{C}$ of all the cellular components in that body or tissue, and may well reflect the predominance of a particular cellular component in that tissue. Most early attempts at tracing diet sources in food web studies, using stable isotopes, were based on the $\delta^{13}\text{C}$ isotopic composition of the bulk animal and its diet. Although an animal's bulk $\delta^{13}\text{C}$ value provides a good index of its carbon assimilation over the long term, it provides little information as to the fate of individual components in the diet. Some metabolites, referred to as essential (some amino and fatty acids for instance) cannot be manufactured *de novo* by the animal and the animal's diet is their only source. The tracking of such essential components provides important information of metabolic pathways that cannot necessarily be inferred from bulk tissue $\delta^{13}\text{C}$ analysis.

Enzymes involved in metabolic processes within the cell tend to fractionate isotopes to different degrees. So some classes of compounds are isotopically different to the bulk $\delta^{13}\text{C}$ value. Consequently, different tissues from an organism are likely to possess slightly different isotopic

compositions from bulk animal $\delta^{13}\text{C}$. Nier and Gulbransen (1939) were the first to report the depletion of lipids relative to whole cell $\delta^{13}\text{C}$. Lipids characteristically have isotopic signatures that are 4-9‰ depleted in $\delta^{13}\text{C}$ compared to the bulk animal $\delta^{13}\text{C}$. In contrast, protein and carbohydrate $\delta^{13}\text{C}$ values are typically similar to the isotopic composition of the bulk animal. Abelson and Hoering (1961) and Park and Epstein (1961) found that protein values were typically 0.1 to 3.9‰ more ^{13}C -enriched compared to bulk tissue $\delta^{13}\text{C}$, whereas carbohydrates were only 0.1 to 1.6‰ enriched. This feature appears to be true of both autotrophs and heterotrophs with the protein fraction being slightly more ^{13}C -enriched than the carbohydrate fraction (DeNiro, 1977). Once metabolic effects are known, it is possible to trace dietary sources of particular metabolites, as their isotopic signatures remain relatively unscrambled through the metabolic pathways. Although it has been known for some time that cellular components have slightly different isotopic compositions compared to their whole body $\delta^{13}\text{C}$, initial isotopic analysis of individual compounds historically involved tedious isolation and purification procedures and bulk tissue $\delta^{13}\text{C}$ analyses were much easier and faster to prepare. Nowadays we have the capability of measuring the isotopic variations at the molecular level to trace nutrient pathways using a technique called compound specific isotope analysis.

1.9 COMPOUND SPECIFIC ISOTOPE ANALYSIS (CSIA)

It has long been a goal of organic geochemists and biochemists to be able to trace individual molecules. The isotopic analysis of individual compounds, compound specific isotope analysis (CSIA) is not a new technique. The analysis of individual compounds began almost forty years ago when pioneering work by Abelson and Hoering (1961) demonstrated that individual amino acids from a protein hydrolysate possessed different $\delta^{13}\text{C}$ values, which covered a range of about 17‰. The most ^{13}C -enriched amino acids were found to be serine, threonine, glycine and aspartic acid, whereas the most ^{13}C -depleted were isoleucine, leucine and the aromatic amino

acids. The carboxyl functions were also generally ^{13}C -enriched by as much as 20‰ relative to the rest of the molecule. The isotopic differences are not surprising if the biosynthesis of amino acids is considered. Intermediates from both the glycolic and TCA pathways provide carbon skeletons for the formation of amino acids via reductive amination or transamination so there are many opportunities for isotopic fractionation along the route. The maximum fractionation between individual fatty acids has been found to be smaller, at around 4‰ (Parker, 1963).

1.9.1 Isotope ratio monitoring-gas chromatography-mass spectrometry

The introduction of sensitive instrumentation to analyse the isotopic composition of individual components in a complex mixture is relatively recent (Matthews and Hayes, 1978; Gilmour *et al.*, 1984; Hayes *et al.*, 1990). The instrumentation most frequently used is a combination of a gas chromatograph linked via a combustion interface to an isotope ratio monitoring mass spectrometer - IRM-GCMS (Matthews and Hayes, 1978; Freedman *et al.*, 1988; Hayes *et al.*, 1990). Studies utilising this technique are thus quite novel and because of its rather embryonic stage there are few existing databases or published literature to consult. Acquisition and processing of data from IRM-GCMS instruments requires careful handling for highly precise and accurate isotope ratio determinations, as analysis of complex mixtures often results in overlapping peaks (Ricci *et al.*, 1994; Merritt *et al.*, 1994; Goodman and Brenna, 1994; Merritt and Hayes, 1994). In addition, derivatization techniques are often required to increase the volatility of the compounds in question so that they can be chromatographed. Many carbon containing compounds contain highly polar functional groups and there are problems with analysing these compounds by gas chromatography (Klee, 1985). It is possible to analyse such compounds without derivatisation, using high polarity stationary phases. However, the phases generally have maximum running temperatures in the region of 250°C, which can lead to long analysis times, broad peaks and reduced resolution, especially for compounds with carbon chains containing in excess of 24 carbon atoms (Klee 1985). Use of low polarity stationary phases (methylsilicone) for the analysis of polar compounds is also generally poor, peak tailing and loss of sample occurs due to adsorption effects, as sample reacts with the stationary phase.

Therefore, the solution to the analysis of these compounds is to derivatize them to less polar derivatives. The derivatization procedure adds additional carbons to a carbon skeleton, and this alters the original isotopic composition of the compound. The derivatization process itself may also be associated with an isotopic effect during the reaction so the isotopic composition of a derivative may be several per mil different to the original compound. The analysis of a six-carbon sugar, for instance, requires derivatisation to form an alditol-acetate and involves the addition of twelve carbon atoms to the sugar. This significantly alters the isotopic composition away from the original sugar $\delta^{13}\text{C}$. Suitable corrections have thus to be made to calculate the original compound $\delta^{13}\text{C}$ value using mass balance equations.

Derivatisation methods may also involve additional isotopic effects which cannot be calculated for from mass balance alone and in such cases it is important to assess isotopic fractionations involved using compound standards of known isotopic compositions. Fatty acids are more amenable to derivatisation since they chromatograph well as methyl esters which involves the addition of only one additional carbon to the original molecule.

1.9.2 IRM-GCMS: description of the Finnigan Delta-S used in this study

Compounds eluted from the gas chromatographic column were combusted to carbon dioxide in the presence of excess oxygen, and the resultant gas removed of any water using a semi permeable membrane before being ionised in the mass spectrometer source. The oxygen was provided from a copper wire within the furnace that is oxidised to copper oxide, an additional platinum wire was also present to reduce nitrous oxides (Fig 1.6).

Masses 44, 45 and 46 were collected simultaneously and integrated $^{13}\text{C}/^{12}\text{C}$ ratios for each compound peak were compared with similar ratios from pulses of reference gas (OGL-C3, $\delta^{13}\text{C} = -39.8\text{‰}$) introduced before and after the sample chromatographic window. Corrections were applied for the ($^{12}\text{C}^{17}\text{O}^{16}\text{O}$) contribution to the mass 45 peak, source linearity and baseline variation during data collection. Since there was a large dynamic range of individual fatty acid abundance, it was important to ensure that the source response was linear over the range 0.2 to 4

volts. At beams of less than 0.1v, the source becomes non-linear and any acids reaching 4 v greatly overloaded the column and caused broad peaks. It was also important to monitor both mass ^{40}Ar and mass 18 (H_2O) at the beginning of the days run to ensure that there were no leaks in the system and that the Nafion membrane was functioning. At the end of a day's running the combustion furnace was left overnight to oxidise with a gentle oxygen stream to replenish the copper oxide.

As mentioned previously corrections were made for the chromatographic background. The 'background' of the chromatogram is essentially that of column bleed and a function of peak resolution. Low bleeds are essential for isotopic analysis, as high bleeds will have huge effects on the isotopic composition of any compound eluted from such a column. IRM-GCMS data reduction was performed using Finnigan MAT Isodat V5.1 and V5.2 software. Results are reported in the conventional delta (δ) notation and are quoted relative to PDB. Background corrections were either performed manually on each peak when using Isodat V5.1 and both manually and automatically using Isodat V5.2 dynamic background correction. Cross calibration of the carbon dioxide 'in house' standard was performed on Sira 24 or VG Prism instruments during the analysis period to ensure consistency of results.

The limiting accuracy and precision for IRM-GCMS analysis was primarily dictated by: (a) Amount of compound injected, peaks of $<0.2\text{v}$ were considered to be too small for reliable data. (b) Extent of coelution with other compounds, only fully resolved peaks gave good reproducible data. (c) Background, GC columns were chosen with low bleeds where possible and complex mixtures were cleaned up of an extraneous material as much as possible before analysis.

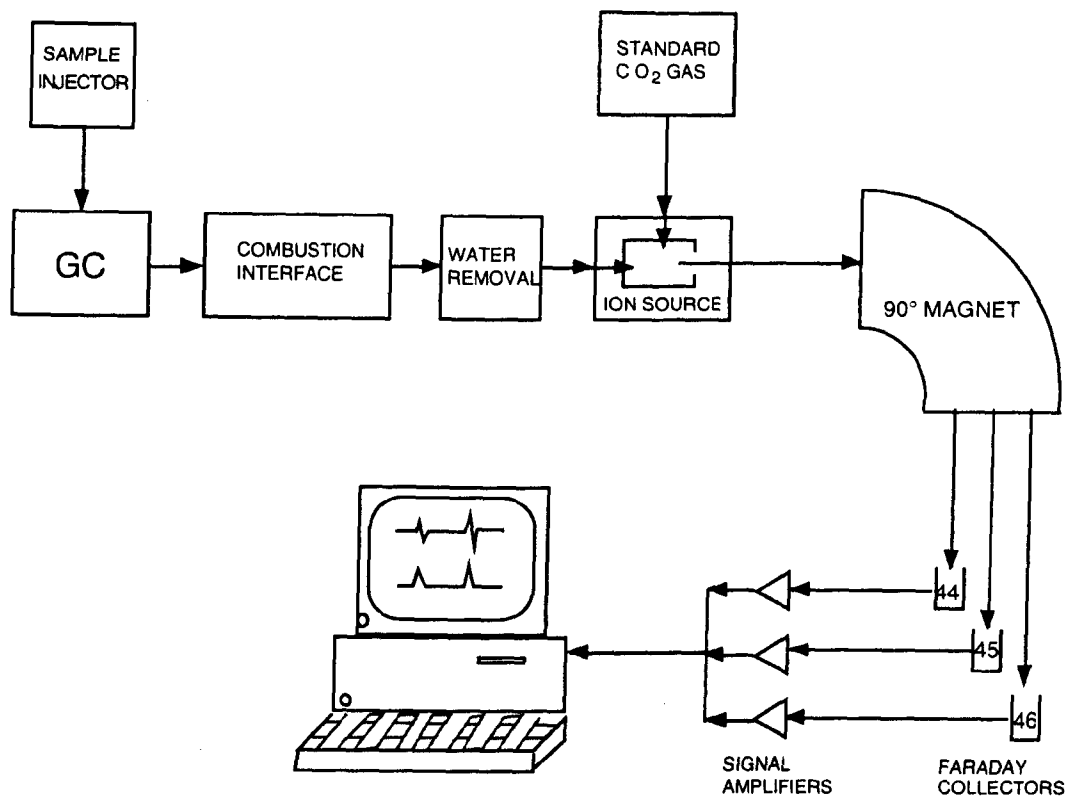


Fig 1.6. Schematic of the IRM-GCMS used in this study, after Hilkert *et al.*, 1992

There are distinct advantages of using IRM-GCMS over the traditional sealed tube bulk combustion of individual compounds: (a) It is possible to get isotopic compositions for several components within a complex mixture in one analysis run. (b) Smaller sample sizes can be measured by IRM-GCMS, sample size for conventional combustion has to be in the microgram range whereas IRM-GCMS can analyse down to nanogram levels. (c) Speed of analysis (d) It is not essential to have totally pure compounds for IRM-GCMS, provided that other compound peaks can be well resolved from the ones in question. This cuts down on the lengthy and tedious purification procedures required for conventional combustion techniques. Any reaction products or contaminants are combusted along with the molecule of interest and this can lead to incorrect isotopic values.

However, there are analytical difficulties with the IRM-GCMS technique. Many compounds of biological interest require derivatisation, which may add several additional carbons to the original molecules altering its original isotopic signature. To date derivatization effects are not

fully understood. Extensive analyses of derivatized standard material in conjunction with analysis of underivatized by conventional gas source mass spectrometry are required to determine isotopic effects before samples can be determined. IRM-GCMS instruments often do not have the facility to identify eluting compounds before combustion, so all samples have to be analysed by GCMS to identify components. This does mean however that sample mixtures can be checked for possible interferences in the sample chromatograms prior to isotopic analysis. It is also important to use gas chromatographic columns that have very low bleeds, as background bleeds can contribute to the isotopic values, high background bleeds cannot be corrected for in chromatograms. Individual compound peaks have to be as well resolved as possible, co-eluting peaks provide isotopic compositions which are a mixture of the isotopic compositions of the individuals.

1.9.3 Applications of IRM-GCMS

Initial studies using this technique were driven by organic geochemists in search of biomarker molecules to characterise sedimentary organic matter to infer paleoenvironments. Despite the aforementioned limitations, IRM-GCMS has already proven valuable in the analysis of individual compounds in this field (for example: Hayes *et al.*, 1990; Jones *et al.*, 1991; Rieley *et al.*, 1991; Mycke *et al.*, 1994). The uses of IRM-GCMS analysis as a tool are very diverse and range from the analysis of organics isolated from meteorites (Gilmour and Pillinger, 1994; Sephton *et al.*, 1998) to the authentication of various flavours (Mosandl *et al.*, 1990), natural oils (Braunsdoft *et al.*, 1993; Spangenberg *et al.*, 1998) and aromas (Bréas *et al.* 1994) in natural product chemistry. Molecular paleontologists have found the technique useful for distinguishing indigenous organic markers in fossil bivalves (CoBabe and Pratt 1995) and the analysis of organic residues in archeological artifacts and lipids extracted from fossil bone have successfully been used to determine dietary sources (Evershed *et al.*, 1994; Evershed *et al.*, 1995). The study of different carbon fixation methods in terrestrial plants (Collister *et al.*, 1994) has benefited from IRM-GCMS, as have studies in metabolic and nutritional research (Hilkert *et al.*, 1992). Compound specific isotope analysis has been used to determine whether a diet is predominantly

marine or terrestrial in Arctic foxes (Pond *et al.*, 1994; Gilmour *et al.*, 1995), polar bears (Hobson and Stirling, 1997) and hominids (Stott and Evershed, 1996). Medical researchers have employed the technique in metabolic studies and in the controversial fields of steroid abuse in sport and meat production (Becchi *et al.*, 1994; Mason *et al.*, 1998). The technique has primarily been used to measure natural abundance stable isotopes, however, it is increasingly being employed for the analysis of slightly enriched species in nutritional studies and biomedicine (Greiner *et al.*, 1996; Brenna, 1997; Menard *et al.*, 1998). Furthermore, the characterisation of environmental pollutants, such as PCBs, has been a recent problem tackled by individual compound isotope analysis (Jarman *et al.*, 1998).

To date a very limited number of studies have involved the use of CSIA to trace metabolites in symbiotic associations. Two studies have addressed the problem of tracing symbiotic signatures in intact associations and have focussed on endosymbioses involving chemosynthetic bacteria in marine bivalves (CoBabe and Pratt, 1995; Abrajano, 1994). The vastly different carbon fixation mechanisms between the chemosynthetic bacteria and that of the carbon fixation of algae, which would form a bivalve's usual filter feeding diet, demonstrate the ability of this technique to pick out individual acids with very different $\delta^{13}\text{C}$ values. Chemosynthetic bacterial carbon fixation provides fatty acids with ^{13}C -depleted values compared to algal fatty acids by as much as 10‰ (Abrajano 1994). The tracing of fatty acids or indeed any other metabolite using CSIA becomes rather more difficult when the possible food sources for the animal are either algae within the water column or possible photosynthetic products from symbiotic algae, living within the host, especially when both algal sources are presumably utilising the C3 photosynthesis pathway. This is the first time this technique has been applied to an algal/invertebrate symbiosis such as in Tridacnidae; initial results were published by Johnston *et al.* (1995).

1.10 SUMMARY OF OBJECTIVES

The main aims of this project were as follows:

1. To develop suitable techniques for the analysis of biochemical compounds to enable the measurement of abundances and isotopic compositions in the *Tridacna*-algal symbioses.
2. To identify translocated algal compounds in the *Tridacnid*/algal symbiosis using specific compound abundances and natural abundance carbon isotopes. In order to do this both symbiotic and non-symbiotic filter feeding bivalves from the same sites were compared to identify isotopic signatures in the host clams that could be attributed to compounds translocated from zooxanthellae. When possible, several animals of a species were analysed to investigate whether there was any significant intraspecies variability in biochemical components or isotopic compositions.
3. To identify a characteristic zooxanthellar compound that can be traced into the host tissues that would enable its use as a biomarker for the presence of zooxanthellar translocation in other algal/invertebrate symbioses.
4. To identify whether the contribution of identified translocated compounds was similar in juvenile and adult clams and whether a diel cycle could be identified in the isotopic composition of specific biochemical components.
5. To investigate whether *Tridacnid* clams precipitate aragonite in isotopic equilibrium with the surrounding water and thus exhibit seasonal changes in their skeletal carbonate. Also to identify any changes in stable isotopic and strontium incorporation when the animals are artificially nutrient supplemented. In doing this establish whether microanalysis of shell carbonate may indicate environmental 'stress' in reef animals.

2 EXPERIMENTAL METHODS

2.1 INTRODUCTION

This chapter describes the general methods used for all samples. Experimental details for specific methods used in subsequent chapters are given in sections 2.5 onwards.

2.2 SAMPLE COLLECTION STRATEGY

All Tridacnid clams and non-symbiotic animals were collected from two sites on the Great Barrier Reef; One Tree Island, a coral cay, situated in the Capricorn group 100 km off the coast of Gladstone, or Orpheus Island, situated approximately 20 km off the coast of Ingham (Figs. 2.1 and 2.2). Orpheus Island is a mainland island surrounded by fringing reef.

The two main species of Tridacnids collected for this study, *T. gigas* and *T. maxima*. *T. gigas* specimens were taken from maricultured stock from Orpheus Island, initially part of an aquaculture project at James Cook University, Townsville funded by the International Centre for Living Aquatic Resource Management (ICLARM).

One clam, *T. gigas*, was held in the JCU aquarium, for approximately 3-6 months, before sacrificing, the aquarium containing water taken from Townsville coastal waters, which is then recirculated. *T. gigas* shells, from a previous nutrient supplementation experiment, using ammonium and phosphate fertilisers, carried out by Belda *et al.* (1993a and 1993b) at Orpheus Island were also acquired. Additional *T. maxima* specimens were also collected under permit from One Tree Island. Several *T. maxima* were collected during the course of an artificial eutrophication experiment at OTI (The Effect of Nutrient Enrichment on Coral Reefs - ENCORE). They were control animals from the project having had no nutrient supplementation (Steven and Larkum 1993). Mantle tissue from *H. hippopus* and tissues from *T. squamosa* were also sampled.

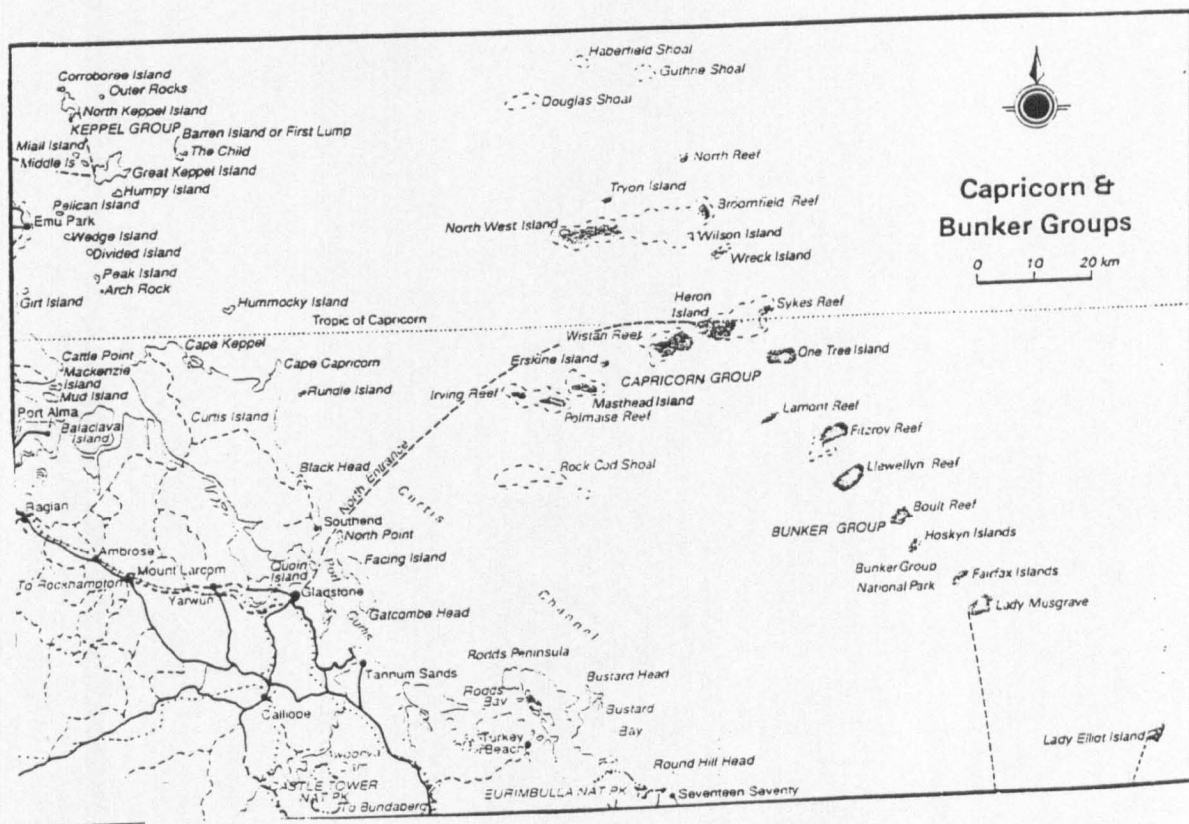


Fig. 2.1 Location map for One Tree Island and Lagoon, Great Barrier Reef, Australia

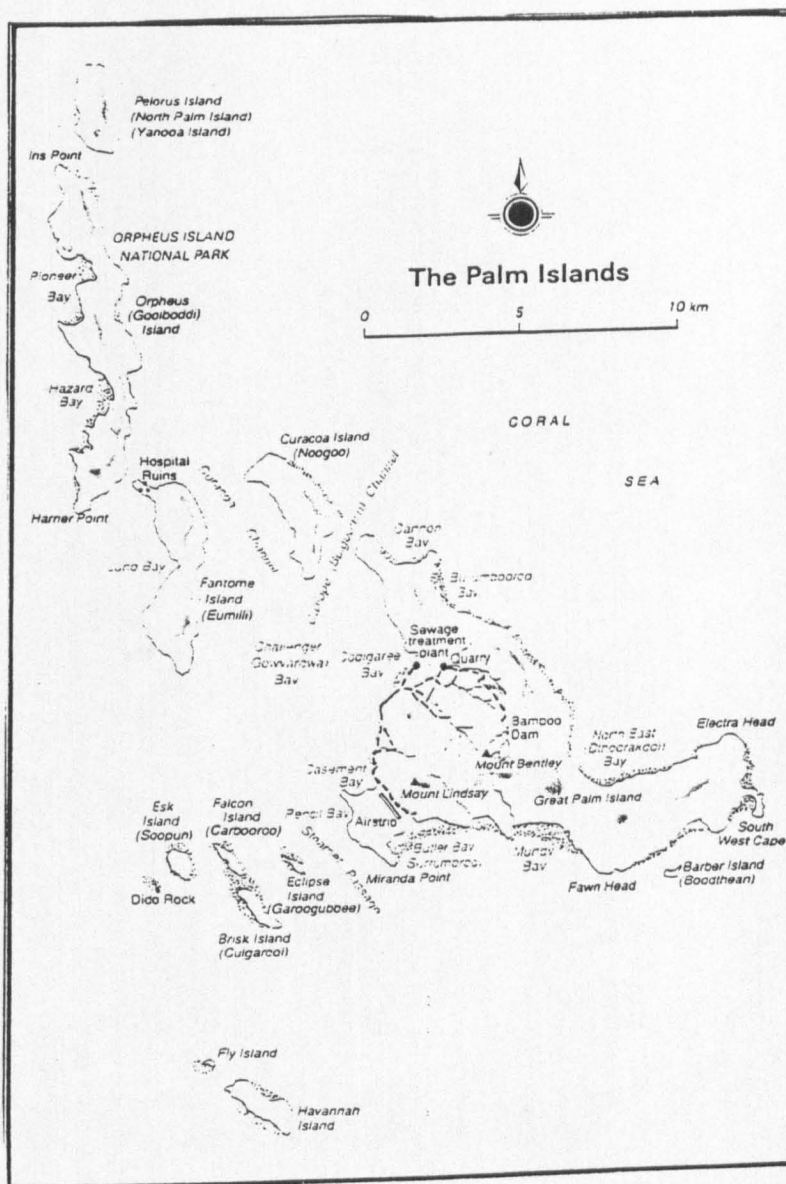


Fig. 2.2 Location map for Orpheus Island, Great Barrier Reef, Australia

All of the non-symbiotic animals, taken for comparison with the symbiotic animals, were filter feeding bivalves which were found at the same sites as the *T. maxima*, from One Tree Island. A summary of symbiotic and non-symbiotic animals collected is given in Table 2.1. Identification and feeding strategies of all species were made by comparison to those in texts (Lamprell 1987; Oliver 1992; Lamprell and Whitehead 1992). Initial identifications were confirmed by John Taylor at the Natural History Museum, London.

2.3 SAMPLE PREPARATION

Before removing clams from their substrate, a small wooden wedge was placed gently between the valves, ensuring that the valves could not close completely. The clam was then removed from the substrate and transported back to the laboratory, the wooden wedge kept in place with string. On arrival in the lab, the animal was shaken gently to remove as much seawater as possible, to minimise the risk of haemolymph contamination. Using a scalpel the adductor muscles were severed from one side of the valves, this allowed the shell to gape open. A clean beaker was held under the animal to collect the haemolymph, and a scalpel was used to cut around the mantle edge. The clam was excised from the shell and dissected to give mantle, adductor, gills, digestive mass and combined remaining tissues. (Fig. 2.3). Smaller animals were excised whole, as were *T. maxima* from the ENCORE project.

Zooxanthellae were prepared from a sample of the mantle tissue using the method of Fitt *et al.*, (1993). Mantle tissue was homogenised in filtered seawater (FSW) (0.45µm) and the homogenate passed through three layers of cheesecloth to remove larger pieces of animal tissue before centrifuging (1500g, 3 min). The zooxanthellae formed a hard pellet while the supernatant, containing host tissue material, was discarded. The zooxanthellae pellet was resuspended in artificial or FSW to wash the algae free from any remaining host cell debris and tissue fluids then re-centrifuged. The washing and centrifugation process was repeated until all host tissue was removed, as confirmed by microscopy.

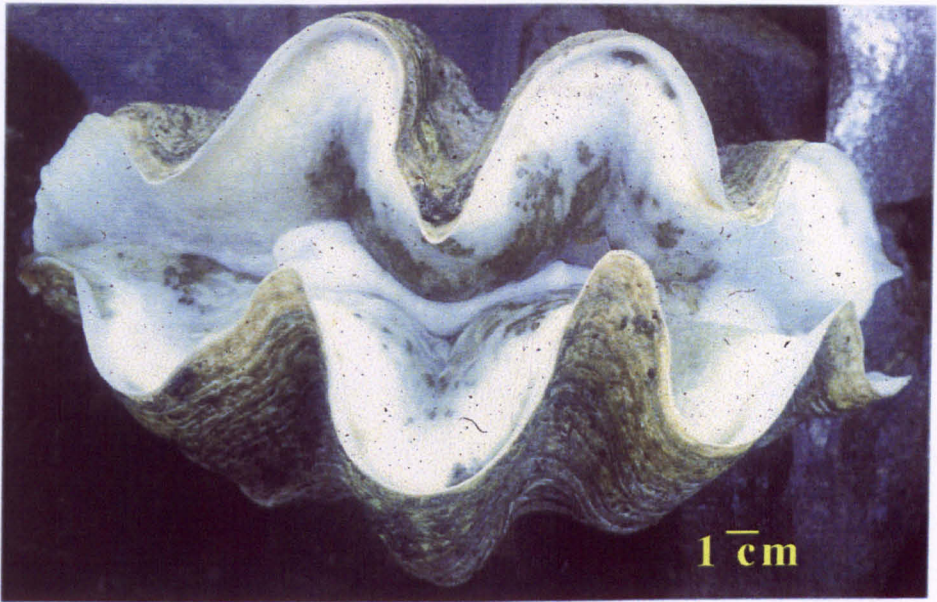


Plate 3. Partial albino *T. gigas*, collected from Pioneer Bay, Orpheus Island.

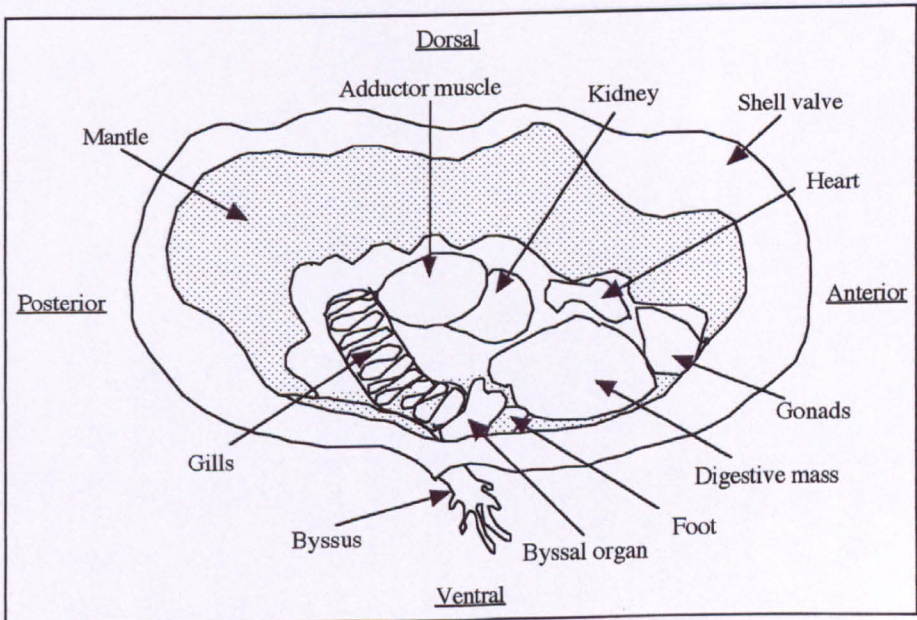


Fig. 2.3 Schematic diagram of the internal organs of *T. gigas*.

Table 2.1. Summary of symbiotic and non-symbiotic animals collected for this study.

<i>Species</i>	<i>Common name</i>	<i>Number collected</i>	<i>Location</i>
Non-symbiotic animals:			
<i>Saccostrea cucullata</i>		28	One Tree Is.
<i>Hyotissa hyotis</i>		2	One Tree Is.
<i>Pinna bicolor</i>	Razor shell	1	One Tree Is.
<i>Pinctada margaritifera</i>		2	One Tree Is.
<i>Septifer bilocularis</i>		3	One Tree Is.
<i>Malleus regula</i>	Hammer shell	2	One Tree Is.
<i>Antigona reticulata</i>		1	One Tree Is.
<i>Barbatia foliata</i>		1	One Tree Is.
<i>Spondylus squamosus</i>	Spiny oyster	1	One Tree Is.
Symbiotic animals:			
<i>Tridacna gigas</i>	Giant clam	10	Orpheus Is.
<i>Tridacna maxima</i>	Giant clam	26	One Tree Is.
<i>Tridacna squamosa</i>	Giant clam	1	One Tree Is.
<i>Hippopus hippopus</i>		1	Orpheus Is.
Shells only:			
<i>Tridacna gigas</i>	Giant clam	20	Orpheus Is.

Non-symbiotic animals were removed from their substrates and excised from their shells in a similar manner, those large enough to yield separate tissues for analysis were dissected, those too small were excised whole. All tissues and zooxanthellae were frozen immediately on excision and transported in dry ice to be freeze-dried at JCU using a commercial refrigeration unit and rotary pump system. Once freeze-dried, the samples were stored at -20°C until required for analysis. Only animals that were devoid of any obvious parasites or infection were chosen for study, this typically meant animals that were free of scar tissue and had mantle tissue that was fully populated with zooxanthellae. Additionally, however, one partial albino *T. gigas*, collected from Orpheus Island was dissected for analysis, for comparison with its zooxanthellate contemporaries (Plate 3). Although environmental stress, such as increased water temperature, has been found to cause bleaching in corals the cause is quite different in giant clams. Norton *et al.* (1995) reported that the zooxanthellar tubules in a bleached individual are atrophied and that this

condition prevents the proliferation of zooxanthellae into some areas of the mantle tissue. Devoid of zooxanthellae, the mantle tissue remains a whitish colour, hence the term albino.

Time constraints did not permit the animals to be held in tanks before sacrificing, which would have enabled them to empty their guts. For those animals in which whole body tissues were taken, the presence of food in their digestive system may present "contamination" of the average animal $\delta^{13}\text{C}$ value when whole body carbon isotope analyses were carried out (DeNiro, 1977). The contribution of gut contents was probably small to whole body in most cases, and is estimated at less than 10-15% of the total body weight.

2.4 SOLVENTS, REAGENTS AND APPARATUS PREPARATION

All solvents used were HPLC grade, purchased from Fisher (UK) or Rathburn Chemicals (UK). Hydrochloric acid (6M) was purified by repeated sub-boiling distillation and any dilutions made from this acid were prepared with high purity grade water. Aristar Sulphuric acid was purchased from Merck (UK) and diluted for use. All glassware used for sample preparation was cleaned, initially with Decon 90, before being roasted at 450°C for 2 h in a muffle furnace to remove any traces of organic material. Glassware that could not be placed in the muffle furnace was treated with chromic acid before rinsing with copious ultra pure water, prepared with Millipore reverse osmosis and MilliQ units. The clean glassware was subsequently stored, wrapped in aluminium foil until required for use. All reagents were checked routinely for contamination throughout the analysis period, new reagents being checked before their routine use, batch numbers were also carefully recorded to ensure isotopic consistency of reagents used for derivatization reactions. Derivatization reagents and other reagents were of analytical quality or better. Polyunsaturated (Matreya PUFA-2 and AOCS FAME std No.3), saturated and odd chain fatty acid standards were purchased from Matreya and Sigma U.K. respectively, 5 ml reaction vials from Supelco (UK) and boron trifluoride-methanol (14%) from Alltech, UK.

Monosaccharide, sugar alcohol, hexose amine and amino acid standards were also purchased from Sigma, as was trifluoroacetic anhydride and acetic anhydride.

2.5 BULK COMBUSTION OF TISSUES FOR $\delta^{13}\text{C}$ ANALYSIS

Approximately half of the samples analysed for bulk $\delta^{13}\text{C}$ were analysed at the Open University employing the technique described below. The remaining samples were analysed using a Micromass Optima (VG Micromass, UK) operating in continuous flow mode with a Carlo Erba Elemental Analyser situated at the University of Vienna. Typical sample size for this system was 0.05 to 0.7mg, loaded into Sn buckets and combusted at 1020°C. Buckets containing no sample typically gave negligible carbon dioxide (<1µg C) compared to sample gas volumes. Reproducibility of $\delta^{13}\text{C}$ data was $\pm 0.2\text{‰}$, based on the repeated analysis of a standard urea powder. A second standard (USGS24) however, reproduced slightly better at $\pm 0.1\text{‰}$.

2.5.1 Bulk $\delta^{13}\text{C}$ analysis as carried out at the Open University

Limited data suggest that treatment of invertebrate tissues with dilute hydrochloric acid should be standard practice (Haines and Montague, 1979). However, treatment of several tissues with hydrochloric acid and sonication had an insignificant effect on subsequent isotopic values in this study and was considered unnecessary. Samples of animal/algal tissues, about 100-500µg, were combusted *in vacuo* using the Dumas sealed tube method (Grady 1982). Quartz tubing and Specpure copper oxide, purchased from Johnson and Mathey UK, were combusted at 900°C before use to remove any traces of organics. Each combustion tube was then “cracked” *in vacuo* and the resultant CO_2 gas exposed to CuO at 450°C (to remove unused oxygen), was purified cryogenically (a) using liquid nitrogen to remove non-condensable gases, (b) using a pentane slush at a temperature of circa -135°C to separate CO_2 from any SO_2 . The volumes of CO_2 sample gases, measured by capacitance manometer, were recorded in order that yields could be calculated to check complete combustion of sample material. The CO_2 gas was then collected into pre-evacuated vessels and analysed immediately using one of two triple collector gas source

mass spectrometers (models: VG Sira 24 and VG Prism). Sub-samples of the NIST standard NBS 21 (graphite) were also combusted and analysed periodically during the study to evaluate the precision of the combustion method.

The replicate analysis of NBS 21 gave $-28.33 \pm 0.05\text{‰}$ ($\delta^{13}\text{C}_{\text{PDB}}$) which was in good agreement with the long-term laboratory value. The triplicate analysis of the fatty acid standard 19:0 (purchased from Sigma) gave $-26.8 \pm 0.1\text{‰}$.

Runs with no sample in the combustion tube gave $0.16\text{ }\mu\text{moles}$ of carbon dioxide or less (as measured by capacitance manometer on the gas purification line). The isotopic composition of the blank was not determined. Since all combusted samples gave $20\text{ }\mu\text{moles}$ of CO_2 , the blank contribution was typically $<1\%$ of the sample and considered negligible. The stocks of PDB have long been exhausted and the in house standard reference gas is actually calibrated to NBS 19, assigning $+1.95\text{‰}$ and -2.2‰ for $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values respectively. The $\delta^{18}\text{O}$ data from combustion experiments were obviously not used, as excess oxygen was supplied by the copper oxide, which would alter the original isotopic signature of the sample. The working standard reference gas, used for the analysis period using the VG Sira 24 and VG Prism, was CO2-5 and has a $\delta^{13}\text{C}$ value of -2.323‰ and $\delta^{18}\text{O}$ of $+8.312\text{‰}$ versus PDB, and was calibrated against NBS 19, assigning $+1.95\text{‰}$ and -2.2‰ for $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ respectively.

2.6 PARTICULATE ORGANIC MATTER (POM)

2.6.1 Pre-combustion of filters

Glass fibre filters, GF/C filters 47mm diameter, were purchased from Whatman Int. Ltd. Glass fibres are commonly used for the collection of POM because they possess high flow rates, have high loading capacities and very low organic blanks when combusted (Altabet 1990). They are suitable for the filtration of suspended solids in all waters as well as the removal of plankton and zooplankton. The filters were combusted at 450°C for 2 hours before use and combusted filters were checked for any trace organics. This cleaning

process was adequate as blank filters produced negligible CO₂ on combustion. Once combusted each filter was weighed and stored in clean aluminium foil, and at no time were the filters handled without the use of gloves.

Water samples (~3 l) were collected into plastic bags from various sites at One Tree Lagoon. The samples were filtered back at the research station, using a millipore multi channel filter holder, linked up to a rotary pump. The particulate samples attached to their preweighed filters were frozen until they could be freeze dried back on the mainland. Freeze dried particulate organic carbon samples were stored at -20°C wrapped in aluminium foil until required for analysis.

2.7 DEVELOPMENT OF LIPID ANALYSIS METHODS

2.7.1 Fatty acid nomenclature

There are several schemes of fatty acid nomenclature currently in existence: (a) trivial names, (b) common nomenclature which uses Greek letters to identify carbon atoms, (c) names derived systematically from the saturated hydrocarbon with the same number of carbon atoms, the final -e being changed to -oic, (d) IUPAC short hand scheme for the number of carbon atoms in a fatty acid chain, the number of double bonds and the position of those bonds relative to the carboxyl carbon. (e) Unsaturated fatty acids can also be described by the location of the last double bond in the chain nearest the methyl end, for instance 18:3Δ^{9,12,15} is an omega-3 (ω³) fatty acid. Nomenclature and common names used in this study are provided in Table 2.2.

Table 2.2. Fatty acid nomenclature used in this study

<i>Systematic name</i>	<i>Trivial name</i>	<i>IUPAC) designation</i>	
Dodecanoic	Lauric	12:0	
Tridecanoic	-	13:0	
Tetradecanoic	Myristic	14:0	
Pentadecanoic	-	15:0	
Hexadecanoic	Palmitic	16:0	
cis-9-hexadecenoic	Palmitoleic	16:1 Δ 9	16:1 ω 7
cis-7-hexadecenoic	-	16:1 Δ 7	16:1 ω 9
Heptadecanoic	Margaric	17:0	
Octadecanoic	Stearic	18:0	
cis-9-octadecenoic	Oleic	18:1 Δ 9	18:1 ω 9
cis-11-octadecenoic	Vaccenic	18:1 Δ 11	18:1 ω 7
Octadecadienoic	Linoleic	18:2 Δ 9,12	18:2 ω 6
Octadecatrienoic	α -Linolenic	18:3 Δ 9,12,15	18:3 ω 3
Octadecatrienoic	γ -Linolenic	18:3 Δ 6,9,12	18:3 ω 6
Nonodecanoic	-	19:0	-
Eicosanoic	Arachidic	20:0	-
Eicosatetraenoic	Arachidonic	20:4 Δ 5,8,11,14	20:4 ω 6
Eicosapentaenoic	-	20:5 Δ 5,8,11,14,17	20:5 ω 3
Heneicadecanoic	-	21:0	-
Docosanoic	Behenic	22:0	-
Docosaheptaenoic	-	22:6 Δ 4,7,10,13,16,19	22:6 ω 3
Tetracosanoic	Lignoceric	24:0	

2.7.2 Extraction of lipids from tissues and zooxanthellae

Lipids can be extracted from tissues by a number of organic solvents, however it is generally believed that no single pure solvent is suitable as a general-purpose lipid extractant. The method outlined by Whyte (1987) and Volkman *et al.* (1993) was chosen to extract the lipids from clam tissues and zooxanthellae, mainly because this method provided three fractions required for this study, one of lipids, a fraction enriched in mono and oligosaccharides and a polysaccharide-containing residue (Fig. 2.3).

10-30 mg of freeze-dried tissues or zooxanthellae were extracted with 2cm³ chloroform/methanol/water (2:4:1) using sonication. The extracts were then centrifuged at 4000 g for 5 minutes and the supernatant extract removed to a clean centrifuge tube. The extraction process was repeated on the residue with further 2cm³ volumes of chloroform/methanol/water until the extracts produced were free of any colour. All extracts from each sample were pooled and separated into two phases by the addition of 5cm³ each of water and chloroform, to form an upper aqueous-methanol layer, rich in mono and oligosaccharides and a lower chloroform layer containing total lipids. The aqueous-methanol layers were removed and concentrated under vacuum at 30°C, stored in a freezer at -20°C, to be processed at a later date for free sugar analysis. The remaining chloroform layers were washed with ultra pure water to remove any polar components, concentrated under nitrogen into pre-weighed vials, dried over anhydrous sodium sulphate and potassium hydroxide overnight, and the total lipid weight recorded (Fig. 2.4) Total lipids were expressed as % lipid per dry weight of sample extracted. Dimethyl sulphoxide used to facilitate the extraction of lipids from various algae by Volkman *et al.* (1993), proved not to be required for the extraction of lipids from zooxanthellae and this reagent was omitted. After freeze drying the zooxanthellae disrupted easily and there was no significant increase in the yield of lipids during its use compared to omitting the reagent.

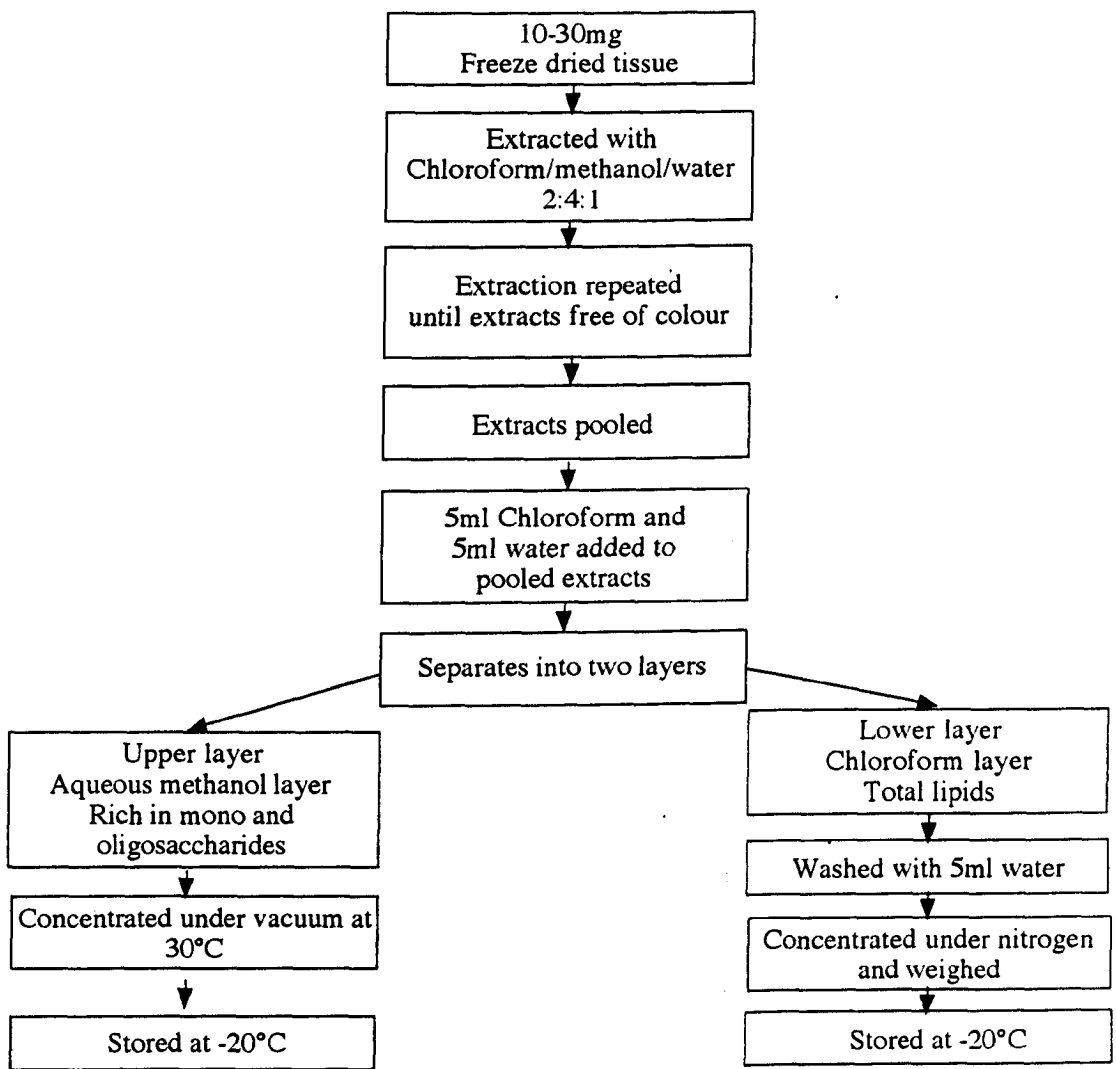


Fig. 2.4 Lipid extraction flowsheet

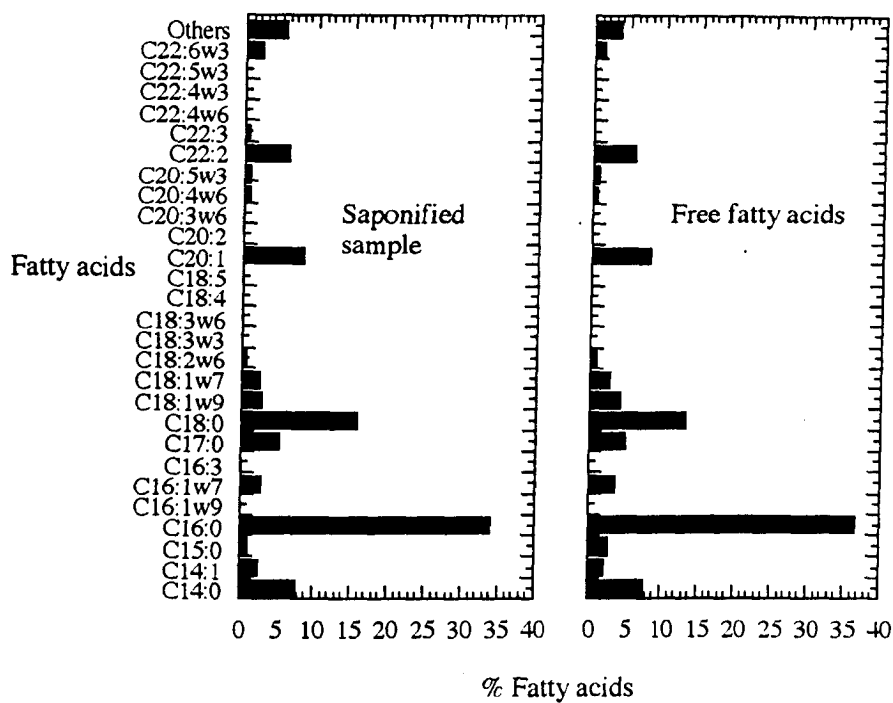


Fig. 2.5 Fatty acid profiles for saponified total lipids and free fatty acids for the non symbiotic bivalve *S. cucullata*.

Total lipid fractions were not saponified for this study since unsaturated acids are particularly sensitive to auto-oxidation in the alkaline solutions used for saponification. For this reason only free fatty acids were studied from the total lipid fraction. The analysis of the total lipids from a sample in which half the sample was saponified and the other half was not, showed that the fatty acid profiles were very similar and that free fatty acid profiles were probably representative of the total lipid fraction (Fig. 2.5). Using nitrogen for evaporation purposes during preparation steps lessened auto-oxidation. Total lipid fractions were stored dry at -20°C until required for derivatization, usually for less than a week. No antioxidant was used for the storage of the lipids, and as long as samples were analysed promptly auto-oxidation proved not to be a problem. Experiments in which the extraction procedure was applied to a blank sample gave no detectable lipids.

2.7.3 Losses of lipid during purification and concentration

According to Dawson and Mopper (1978) there can be considerable losses of monosaccharides, amino sugars and amino acids onto the glass surfaces of equipment used for extraction and purification. The losses were thought to be due to wall induced condensation reactions with other hydrophilic co-extracted components. They found that the addition of 50% glycerin in ethanol solution to the extract during the concentration steps reduced the random losses of natural compounds by coating the glass walls and preventing the condensation reactions. The presence of fatty acid moieties were thought to perform the same function as the glycerin. This finding posed the question of whether there were significant losses of lipid onto the glass equipment used in this study. The use of polypropylene vessels, which have less adsorptive properties, was not possible for this study as plastizers are easily leached from plastics and could interfere with subsequent GCMS and IRM-GCMS analysis. To investigate how much lipid or derivatized lipid material was being lost on glass surfaces, a selection of glassware used in the purification steps was checked by weight for residual lipid or derivatives after they had supposedly

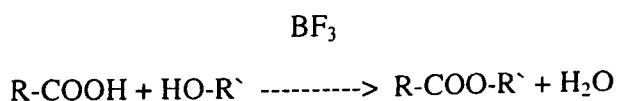
been removed (Table 2.3). Losses were found to be typically 10% or less and provided sufficient sample was extracted the reduction in recovery was not found to be a problem. Also no preferential losses of individual fatty acids were noted. To minimise lipid losses for small sample sizes, the derivatization procedure was carried out in the vials used for lipid storage, provided a teflon lined septa was used in the vial lid.

Table 2.3. Lipid losses during sample preparation.

<i>Sample</i>	<i>Weight of FAMES</i> (g)	<i>Residue weight</i> (g)	<i>Sample left on glass</i> %
T.gigas mantle	0.00044	0.00001	2.3
T.gigas mantle	0.00093	0.00010	10.8
T.gigas mantle	0.00095	0.00007	7.4
T.gigas mantle	0.00070	0.00007	10.0

2.7.4 Derivatization of fatty acids

Although there are several derivatization methods for fatty acids, Fatty Acid Methyl Esters (FAMES) are the classical derivatives of fatty acids and carboxylic acids for gas chromatography. Fatty acids themselves are often too polar to chromatograph well and can cause tailing because of non-specific reactions with the column lining, whereas fatty acid methyl esters are non polar, are more volatile than their respective acids and chromatograph well (Knapp 1979). Boron trifluoride/methanol derivatization was chosen for this study because of its ease of use and also because pilot experiments showed no obvious loss of polyunsaturated acids on derivatization. The derivatization of fatty acids using boron trifluoride methanol involves the addition of a methyl group onto the fatty acid chain forming the fatty acid methyl ester. The process is that of esterification and involves the condensation of the carboxyl group of the fatty acid and the hydroxyl group of the methanol, with the elimination of water. Boron trifluoride serves as a catalyst in the reaction:



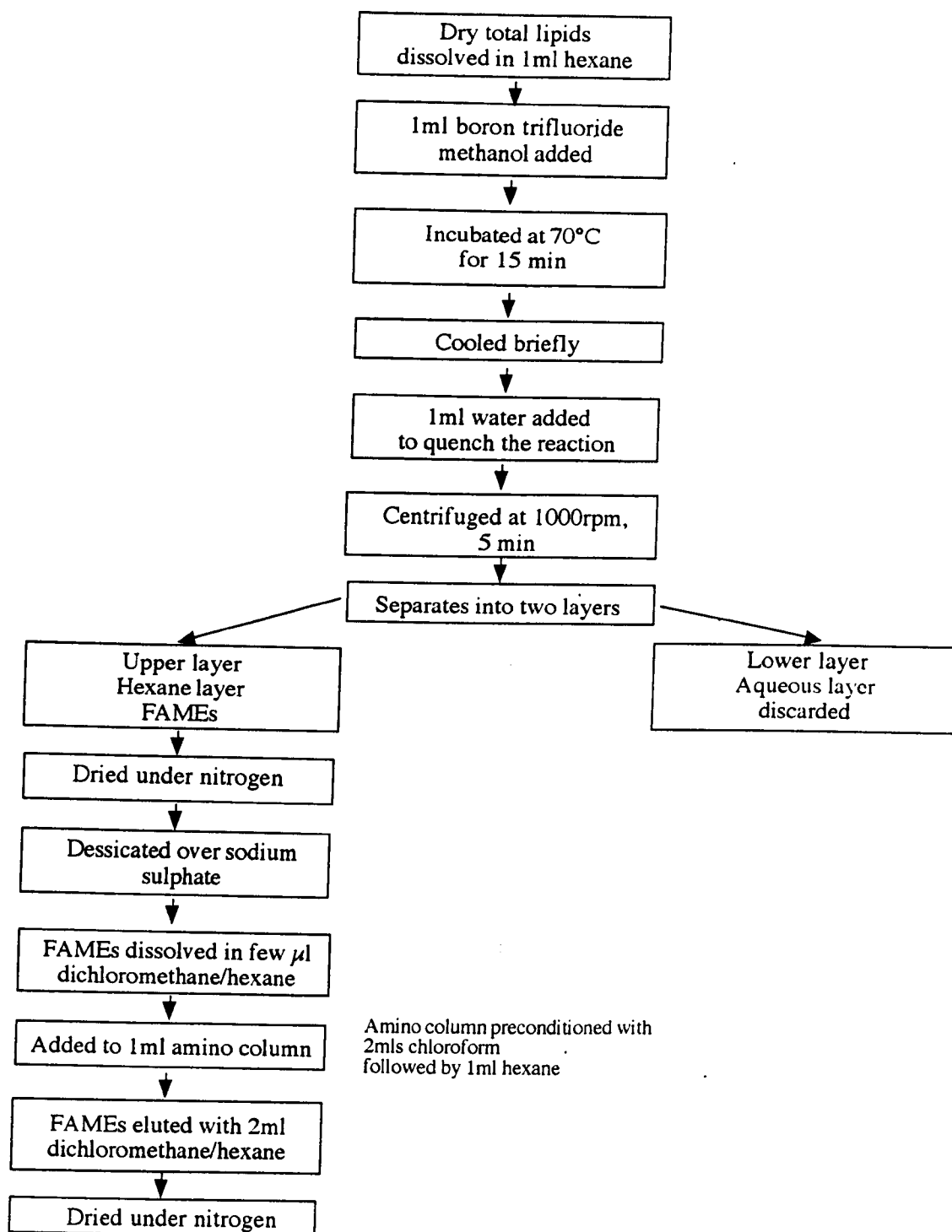


Fig. 2.6 Fatty acid derivatisation and FAME cleanup flowchart

Methyl esters were also chosen primarily for the isotopic analysis of fatty acids because only one carbon atom is added to the fatty acid chain during the derivatization reaction, causing minimal change to the original isotopic signature. Dimethoxypropane, commonly used as a water scavenger was omitted, since this reagent reacts in acid solution to yield acetone and methanol. A second source of methanol in the reaction could disrupt simple isotopic mass balance equations for subsequent $\delta^{13}\text{C}$ determinations.

2.7.5 Derivatization and FAME solid phase clean up

Dry total lipid fractions were dissolved in 1 cm³ of hexane and transferred to 5 cm³ reaction vials along with 1 cm³ of 14% boron trifluoride-methanol. Using a heating block the vials were incubated at 70°C for 15 minutes. After brief cooling 1 cm³ of water was added to quench the reaction and the vials were centrifuged at 200g for 5 minutes to facilitate phase separation. The upper hexane layers containing the fatty acid methyl esters (FAMEs) were removed into pre weighed vials, dried under a stream of nitrogen and subsequently desiccated over anhydrous sodium sulphate and potassium hydroxide overnight (Fig. 2.6.)

The FAMEs, typically small oily beads of liquid in the base of the vial, were cleaned up of any residual non-FAME material using solid phase extraction with 1cm³ amino (NH₂) solid phase columns (purchased from Alltech, UK), preconditioned with 2cm³ chloroform followed by 1cm³ hexane. It was important to remove the bulk of any plant pigments, remaining from the extraction at this stage, as they tended to interfere with the FAME peaks in subsequent GCMS and IRM-GCMS analysis. The derivatized lipids were dissolved in a few μl of dichloromethane/hexane (3:1) and added to the preconditioned column. The FAMEs were then eluted off the column with 2cm³ of dichloromethane/hexane (3:1) and dried under a stream of nitrogen (Fig. 2.6). The FAMEs were typically analysed within 24 hours and until analysed they were flooded with nitrogen and stored dry at -20°C.

Table 2.4 Operating conditions for GCMS and GCirmMS analysis of FAMES using BPX 70 columns.

Condition file	Column type	Analysis type	Film thickness (microns)	Internal diameter (mm)	Length (m)	Mode of injection	Carrier gas, head pressure	Column flow rate, ml/min
1	BPX 70	GCMS	0.25	0.32	50	split 10:1	He, 25psi	1
2	BPX 70	GCMS	0.25	0.2	50	split 10:1	He, 25psi	1
3	BPX 70	GCMS	0.25	0.2	30	split 10:1	He, 12psi	1
4	BPX 70	GCirmMS	0.25	0.32	50	on-column	He, 25psi	1
Condition file	Initial oven temperature degrees C	Hold time min	Final oven temperature degrees C	Ramp rate degrees C/min	Hold time min	Intermediate temperature degrees C	Ramp rate degrees C/min	Hold time min
1	140	1	210	2	4			
2	140	1	210	2	14			
3	140	1	210	2	0			
4	50	2	160	20	2	220	1.5	5

Several saturated odd chain fatty acid standards (9:0, 11:0, 13:0, 15:0, 17:0, 19:0, 21:0 and 23:0) were also derivatized and used as aids for identification and for use in mass balance calculations for isotopic analyses. Yields, measured by using internal standard addition before and after elution, from solid phase cleanup columns for fatty acid methyl esters were typically 90% or greater as indicated from those of saturated fatty acid standards.

2.8 MASS SPECTROMETRIC ANALYSIS OF FATTY ACID METHYL ESTERS

Fatty acid methyl esters were analysed by Gas Chromatography Mass Spectrometry (GCMS) and isotope ratio monitoring Gas Chromatography Mass Spectrometry (IRM-GCMS) using BPX70 columns purchased from SGE.

2.8.1 GCMS analysis of FAMES

FAMES were dissolved in 50 μ l hexane and analysed on a Hewlett-Packard 5890 GCMS coupled to a 5791 Mass Selective Detector (MSD) Column details and temperature programs are given in Table 2.4.

Identities of fatty acids were made on the basis of comparison with retention times of known standards and by comparison of their mass spectra with library spectra using a NIST/EPA/MSDC database. Typical MSD operating conditions were electron multiplier 1700-2500 volts, electron impact energy of 70 eV and mass spectra were acquired from 50 to 500 daltons at a rate of 0.8 scans s⁻¹ using total ion monitoring. Quantitation was performed using Hewlett-Packard Chemstation software, with fatty acids being reported as per cent of total fatty acids. Positional isomers of several of the acids were established, however geometric isomers were not characterised.

2.8.2 IRM-GCMS analysis of FAMES.

Once fatty acid identities were established by GCMS, the carbon isotopic compositions of the individual FAMES were determined by Isotope Ratio Monitoring Gas Chromatography Mass Spectrometry (IRM-GCMS) using a Finnigan MAT Delta S/GC using on column

injection onto a BPX-70 column, conditions given in Table 2.4. This system has been described previously by Merritt *et al.*, 1994 and a schematic diagram of the machine was given in chapter 1, Fig. 1.5. A capillary gas chromatograph (Varian 30400) is coupled to a Finnigan Delta/S via a combustion interface. Compounds eluting from the capillary column are combusted, water removed by a Nafion membrane and pass to the ion source of the mass spectrometer. The isotopic ratios of all sample peaks are related to a standard CO₂ gas (introduced as three pulses before and after the sample peaks) by sample integration between the pulses.

Background correction

The signal corresponding to each sample peak is mix of several components (Merritt and Hayes 1994):

$$i_t = i_s + i_b$$

$$i_b = i_e + i_l + i_r + i_i$$

where *i* refers to the ion current and the subscripts defined as follows: *t* = total, *s* = sample related, *b* = background, *e* = electronic offset, *I* = impurities in carrier gas stream, *r* = residual gases in ion source and *l* = air leaks. Background ion currents are thus measured and subtracted from the sample peak during data collection. This is particularly important for columns that have significant column bleed. For the analysis of the fatty acid methyl esters, for both standards and samples, the BPX70 columns had relatively low bleeds so the background was fairly clean and the background correction required for sample analysis was minimal. Generally background corrections applied by the Isodat software were adequate, however it was occasionally necessary to manually edit the files to define peaks and background levels for selected peaks, especially if backgrounds were rapidly changing due to column bleed or underlying unresolved components. Evaluation of background correction techniques has been covered elsewhere (Ricci et al 1994).

Standard reproducibility

The reproducibility of the IRM-GCMS FAME analysis was tested by repeated analyses of a fatty acid standard mixture, PUFA-2 (Matreya,UK) and a simple mix of saturated odd chain fatty acids (15:0, 17:0, 19:0, 21:0 and 23:0). External reproducibility, based on the running of PUFA-2 and saturated fatty acid standards, was 0.1-4.2‰ (1 σ) depending on how well the peaks were resolved, only those extremely well resolved peaks gave a reproducibility of $\leq 0.3\%$, 1 σ . (Table 2.5 and Table 2.6). Although the long term isotopic composition of PUFA-2 appears to change over time, as the isotopic composition of some of the fatty acids shifts by as much as 4‰, the short-term reproducibility is primarily dictated by the resolution of the peaks, with small poorly resolved peaks giving poor reproducibility. The long-term change may be related to auto-oxidation of the standard, however, this merits further investigation. All related sample analyses (i.e. tissues from one animal) were thus carried out as soon as possible after derivatisation, minimising any storage time.

Background corrected data was more reproducible than raw data generated by the Isodat software package and in general manually correcting the data was better than the Isodat dynamic correction routine for saturated odd chain acids 13:0 to 23:0 (Table 2.5). Well resolved acids such as these reproduced to 0.3‰ or better. PUFA II fatty acids were less reproducible, errors for all the acids analysed ranged from 0.2-4.2‰ and typically well-resolved peaks reproduced to $<1\%$ (Table 2.6).

Table 2.5. FAME $\delta^{13}\text{C}$ values (‰) of the odd saturated acids, 13:0 to 23:0. Raw and background corrected data (errors $\pm 1\sigma$, n=3-4).

<i>Fatty acid</i>	<i>Raw data</i>	<i>Background corrected data - manual</i>	<i>Background corrected data - software</i>
13:0	-29.8 \pm 0.2	-30.0 \pm 0.1	-29.7 \pm 0.5
15:0	-30.6 \pm 0.1	-30.9 \pm 0.3	-30.6 \pm 0.5
17:0	-31.9 \pm 2.0	-29.5 \pm 0.3	-29.6 \pm 0.4
19:0	-28.6 \pm 1.5	-27.2 \pm 0.3	-27.2 \pm 0.3
21:0	-27.9 \pm 3.0	-25.1 \pm 0.1	-25.0 \pm 0.2
23:0	-23.4 \pm 0.6	-24.0 \pm 1.0	-22.7 \pm 0.6

Table 2.6. FAME $\delta^{13}\text{C}$ values (‰) of fatty acids of the PUFA-2 standard analysed between November 1993 and November 1994. Errors $\pm 1\sigma$

<i>Fatty acid</i>	<i>Nov 93</i>	<i>Dec 93</i>	<i>Feb 94 - Nov 94</i>
16:0	-18.1 \pm 0.6	-20.1 \pm 1.2	-17.4 \pm 0.8
16:1 ω 9			-15.4 \pm 1.2
16:1 ω 7	-29.0 \pm 1.5	-25.1 \pm 2.1	-21.7 \pm 2.5
18:1 ω 9	-14.5 \pm 0.2	-15.8 \pm 1.4	-15.4 \pm 1.0
18:1 ω 7	-25.1 \pm 0.4	-26.6 \pm 2.1	-24.0 \pm 1.2
18:1 ω 9+7	-17.5	-17.0 \pm 1.2	-17.2 \pm 0.9
18:2 ω 6	-25.1 \pm 1.0	-23.0 \pm 1.9	-22.9 \pm 2.3
18:3 ω 6	-39.1 \pm 1.0	-38.1 \pm 1.6	-35.0 \pm 4.2
20:4 ω 6	-22.6 \pm 0.5	-22.8 \pm 1.5	-22.5 \pm 2.3
20:5 ω 3	-31.7 \pm 1.1	-29.4 \pm 1.5	-26.0 \pm 1.7
22:4 ω 6	-24.9 \pm 1.0	-22.7 \pm 2.2	-20.8 \pm 1.3
22:5 ω 3	-28.9	-29.9 \pm 2.5	-25.8 \pm 2.2
22:6 ω 3	-29.7 \pm 1.3	-29.5 \pm 1.8	-28.4 \pm 1.3
	n=7	n=15	n=11

IRM-GCMS Peak overloading experiments

Experiments on peak overloading were undertaken since the dynamic ranges of the FAMES within each sample were known to be large from GCMS traces. Overloading the GC column with the major peaks was often necessary to determine the $\delta^{13}\text{C}$ values of minor peaks. Varying concentrations of the saturated fatty acid standard 19:0 were

analysed (31 -1019 ng μl^{-1}) by IRM-GCMS. $\delta^{13}\text{C}$ values for those standard concentrations that produced Gaussian peaks The $\delta^{13}\text{C}$ values for Gaussian peaks versus overloaded peaks were within ± 2 error at $-27.6 \pm 0.3\text{‰}$ and $-27.8 \pm 0.1\text{‰}$ respectively (errors $\pm 1\sigma$) (Table 2.7).

The data demonstrate the beneficial effect that background correction has on the data in reducing the standard deviations on both Gaussian and non-Gaussian peaks and confirms the findings of Gilmour and Pillinger (1994). Source linearity was typically carried out between 0.2 and 4.0 v/s and sample peaks were maintained, as much as was practicable, within this range. The reproducibility of the standard 19:0 acid in this range was $-27.5 \pm 0.2\text{‰}$, (1σ , background corrected).

Table 2.7. Carbon isotopic values for a saturated odd chain fatty acid, 19:0 measured at different concentrations (31 to 1019ng/μl).

<i>Concentration ng/μl (19:0 acid)</i>	<i>δ¹³C, Isodat background corrected</i>	<i>δ¹³C, manually background corrected</i>	<i>Peak shape type</i>	<i>Area (v/s)</i>
31	-26.8	-27.7	Gaussian	0.6
61	-27.9	-27.2	Gaussian	1.3
61	-27.4	-27.4	Gaussian	1.6
121	-27.8	-27.7	Gaussian	3.7
121	-27.8	-27.6	Gaussian	4
242	-27.6	-28	Gaussian	6.3
364	-27.8	-27.9	Gaussian	9.2
485	-27.8	-28	non-Gaussian	8.14
606	-27.9	-27.6	non Gaussian	8.8
606	-28.1	-27.9	non Gaussian	11.5
970	-27.7	-27.8	non Gaussian	13.4
1019	-27.7	-27.8	non Gaussian	28.6
Average total	-27.7	-27.7		
1 std deviation	0.3	0.2		
Average Gaussian	-27.6	-27.6		
1 std deviation	0.4	0.3		
Average non Gaussian	-27.8	-27.8		
1 std deviation	0.2	0.1		
Average over normal analysis area <4 v/s	-27.5 0.5	-27.5 0.2		

Auto-oxidation experiments

Auto-oxidation can be a significant problem with the analysis of fatty acids, especially if the samples are stored without an antioxidant and at temperatures exceeding -20°C. It is important to know that the isotopic signature of an acid does not change with time, especially if this is a saturated acid remaining after auto-oxidation. Experiments were carried out to investigate the effects of auto-oxidation both on the fatty acid profile of a sample and the isotopic compositions of its individual fatty acids. Six samples were split into two halves, one half being derivatized and analysed immediately for fatty acid abundances, the second half being left at 20°C, without an antioxidant, for 7 days, before analysis. Isotopic analysis was carried out on four pairs of these samples. Results confirm

that unsaturated fatty acids are rapidly lost when left at 20°C for one week as seen in Fig.2.7, whilst the relative concentrations of saturated fatty acids in the samples increases after auto-oxidation. However the isotopic signatures of many of the saturated acids, left after this treatment, possess similar $\delta^{13}\text{C}$ values as those that were analysed immediately (Table 2.8), most FAMES being within 1 sigma errors. The 17:0 acids however are quite different isotopically between the two treatments in three of the four cases analysed. Since odd chain fatty acids are often an indication of bacterial contamination it is possible that the isotopic 15:0 and 17:0 signatures are due to bacterial degradation of the sample in addition to the oxidation effects. These findings are also important for the interpretation of results from the analysis of lipid material, which will naturally have undergone auto-oxidation, such as lipids in sediments, or within POM in the water column. Previous studies of lipid material trapped within shell material (CoBabe and Pratt 1995)) have assumed that such oxidation processes do not change the isotopic signature of fatty acids and the above experiments confirm this to be a valid assumption within analytical errors for the even chain fatty acids. The change in the isotopic composition of the odd chain fatty acids, however, merits further investigation.

Fig.2.7 Effect of auto-oxidation on the fatty acid profiles and isotopic values for saturated acids. Samples were either derivatised immediately or left at 20°C for one week before analysis.

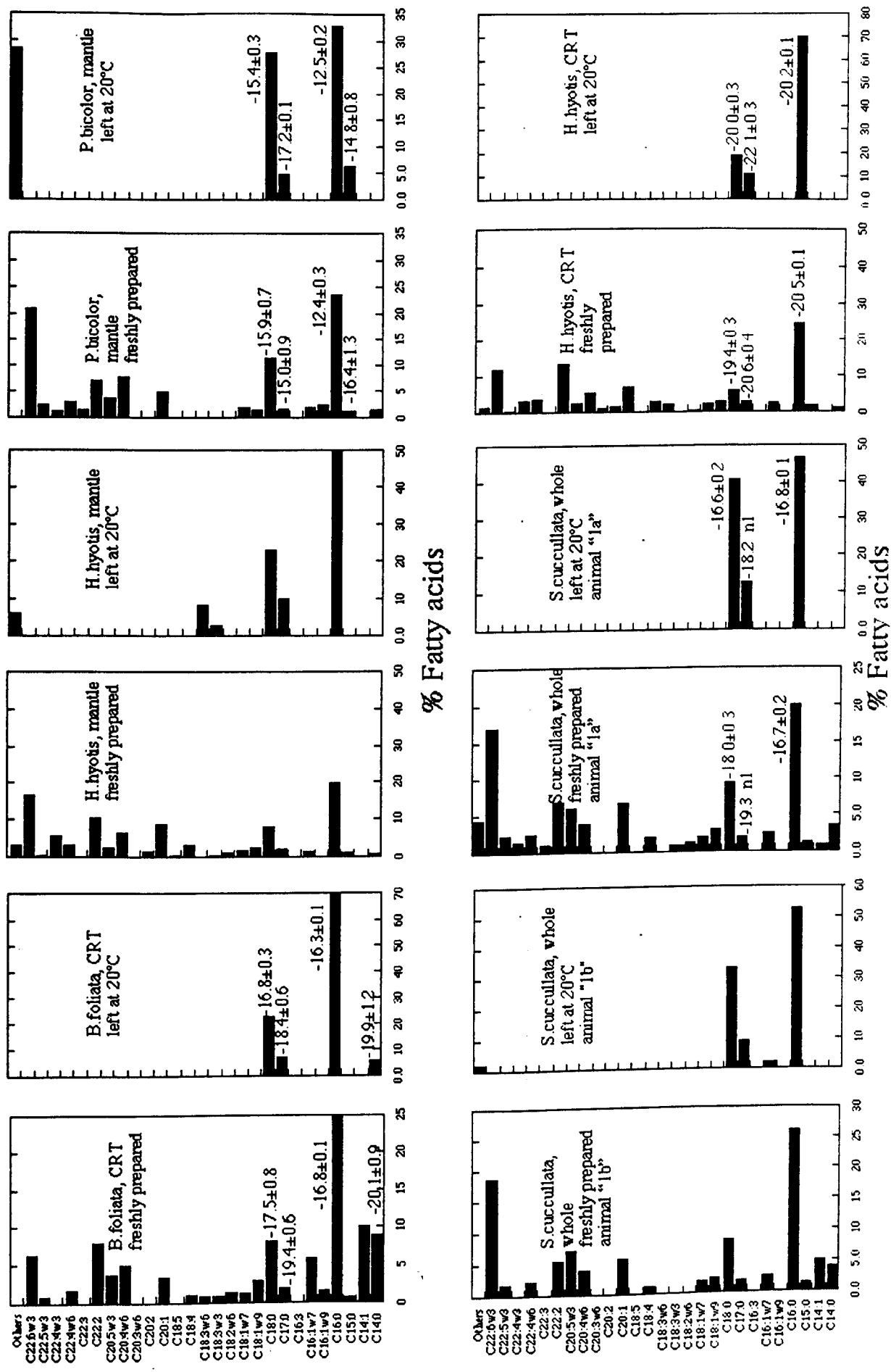


Table 2.8. Carbon isotopic compositions of fatty acids in samples which were either prepared immediately ('fresh') or left at 20°C for 7 days before derivatization. Isotopic values are expressed as $\delta^{13}\text{C}_{\text{PDB}}(\text{‰})$. Errors are 1σ . When insufficient analyses were completed for a statistical average and standard deviation to be calculated the number of analyses is quoted.

Fatty Acid	Left at 20°C	$\pm 1\sigma$	Fresh	$\pm 1\sigma$	Δ difference
	<i>H. hyotis</i> CRT		<i>H. hyotis</i> CRT		<i>H. hyotis</i>
14:0	nd		nd		
15:0	nd		nd		
16:0	-20.2	n2	-19.3	0.5	-0.9
17:0	-22.1	n2	-20.1	1.5	-2.0
18:0	-20.0	n2	-20.3	0.4	0.3
	<i>B. foliata</i> CRT		<i>B. foliata</i> CRT		<i>B. foliata</i>
14:0	-19.9	1.2	-20.1	n2	0.2
15:0					
16:0	-16.3	0.1	-16.8	n2	0.5
17:0	-18.4	0.6	-19.4	n2	1.0
18:0	-16.8	0.3	-17.5	n2	0.7
	<i>P. bicolor</i> mantle		<i>P. bicolor</i> mantle		<i>P. bicolor</i>
14:0			-17.3	0.3	
15:0	-14.8	0.8	-16.4	1.3	1.6
16:0	-12.5	0.2	-12.0	0.3	-0.5
17:0	-17.2	0.1	-15.0	0.9	-2.2
18:0	-15.4	0.3	-15.9	0.7	0.5
	<i>S. cuccullata</i> whole		<i>S. cuccullata</i> whole		<i>S. cuccullata</i>
14:0			-16.4	n2	
15:0					
16:0	-16.8	0.1	-16.7	0.2	-0.1
17:0	-18.2	n1	-19.3	n1	1.1
18:0	-16.6	n2	-18.0	n2	1.4

Quantitation and data manipulation

The addition of an extra carbon from the methanol during the esterification reaction alters the original isotopic signature of the acid, however methanol is always provided in excess

during the reaction and consequently it is assumed that no appreciable kinetic isotope effect is associated with the derivatization. In order to confirm this assumption, underivatized odd chain saturated fatty acids (13:0, 15:0, 17:0, 19:0, 21:0 and 23:0) and their derivatives were separately combusted, the resultant CO₂ gases purified cryogenically, and subsequently analysed on a dynamic stable isotope mass spec (VG Sira), following the sealed tube method described in the general methodology (Grady *et al.* 1982). The derivatives were also analysed by IRM-GCMS.

The derivatization of the fatty acid standards produced fatty acid methyl esters which were isotopically lighter than their corresponding fatty acids, thus indicating that the methanol within the boron trifluoride/methanol reagent was isotopically lighter than the fatty acids, pulling the isotopic signature of the derivatives down (Fig. 2.8). It is possible to calculate the isotopic signature of the methanol by using mass balance equations, knowing the isotopic signature of the fatty acid standards and their derivatives, and use the calculated methanol value to calculate the isotopic compositions of original fatty acids from samples from their derivative values. However, because of the errors inherent in dealing with very small fractions and as small deviations in the fraction can make a huge difference in the calculated $\delta^{13}\text{C}$ value, it was decided that the bulk combustion of the derivatization reagent boron trifluoride methanol alone, to get an isotopic value for the methanol would be preferable for use in mass balance equations for sample analysis, with the proviso that there was no isotopic fractionation associated with the derivatization reaction itself and that the derivatization method was reproducible. Sub-microlitre quantities of boron trifluoride methanol were also injected into the IRM-GCMS to get a $\delta^{13}\text{C}$ value for the methanol. The batch of derivatization reagent used to prepare the FAMES of the odd chain saturated standards gave a $\delta^{13}\text{C}_{\text{methanol}}$ value of $-42.1 \pm 0.1\text{‰}$ (1σ , $n=3$).

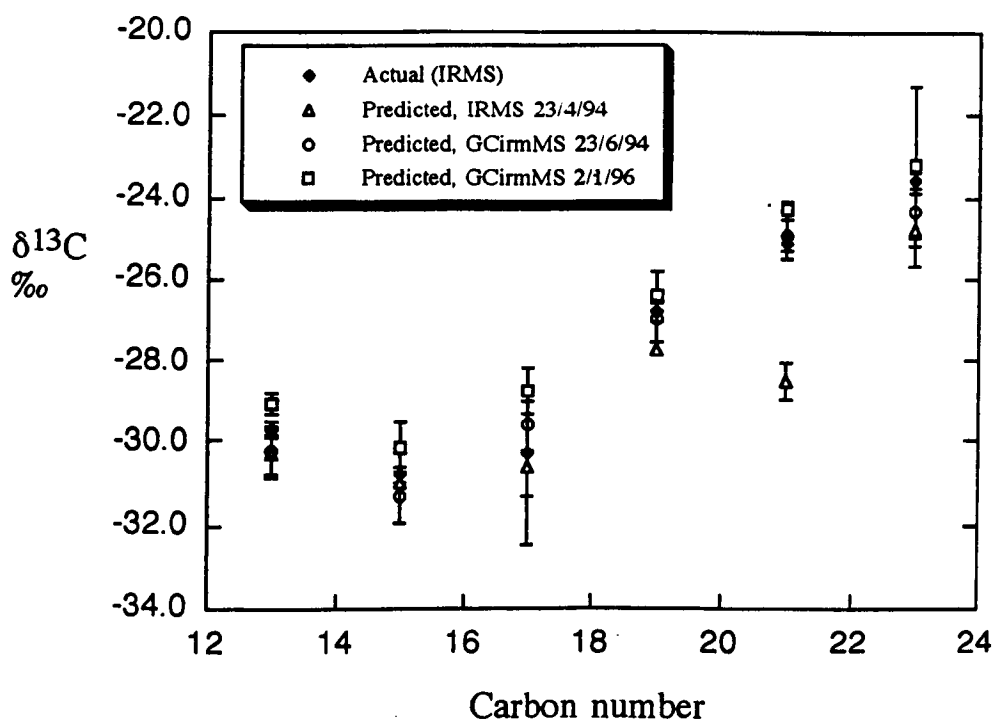


Fig. 2.8 Comparison of fatty acid $\delta^{13}\text{C}$ values to those predicted by mass balance equations using the $\delta^{13}\text{C}$ of their respective FAMES analysed by IRMS and IRM-GCMS. ($\delta^{13}\text{C}_{\text{methanol}} = -42.1 \pm 0.1$, error bars 2σ)

Thus mass balance equations can be used to calculate the isotopic composition of the original fatty acid provided the isotopic composition of the added carbon, from the methanol is known:

$$\text{Fatty acid } \delta^{13}\text{C } \text{‰} = ((n+1 \times \text{FAME } \delta^{13}\text{C } \text{‰}) - (\text{BF}_3\text{MeOH } \delta^{13}\text{C } \text{‰})) / n$$

Where n = the number of carbon atoms in the original fatty acid chain.

Predicted results agree with actual $\delta^{13}\text{C}$ values for the original fatty acids within 2σ , apart from the IRMS analysis of the 21:0 FAME (Fig. 2.8.). The results are reproducible, as demonstrated by repetition of this experiment (Fig. 2.8) and confirm that there is no appreciable isotopic fractionation associated with the derivatization procedure. The results from this study agree with the findings of Abrajano *et al.* (1994).

It is important to discuss at this point the anomalous data point for the 21:0 predicted value. The discrepancy between the predicted fatty acid $\delta^{13}\text{C}$ composition of FAME 21:0 analysed by IRMS appears to be absent when the same FAME was analysed by IRM-GCMS. A major problem with the bulk combustion of any sample is that everything that is loaded into the quartz tube will indeed be combusted to CO_2 , so any small contaminant will be combusted too. The discrepancy can thus be explained by a minor contaminant in the FAME solution that was later confirmed by analysis by GCMS. Analysis of FAMES using IRM-GCMS provided chromatograms that could be checked carefully for any co-eluting contaminants and thus was preferred to bulk analysis for mass balance equation data and was also more convenient. It was essential to recheck isotopic effects if different derivatization reagents were used, as different boron trifluoride methanol batches were found to vary by as much as 17‰ for the $\delta^{13}\text{C}_{\text{methanol}}$. In total three different batches of Boron Trifluoride-Methanol were used during this study, one purchased from Sigma (UK) and two from Alltech (UK), which had $\delta^{13}\text{C}_{\text{methanol}}$ values of $-42.1 \pm 0.1\text{‰}$, $-53.1 \pm 0.1\text{‰}$ and $-59.1 \pm 0.1\text{‰}$ respectively.

2.9 ENZYMATIC TECHNIQUES FOR THE MEASUREMENT OF GLUCOSE AND GLYCEROL LEVELS IN HAEMOLYMPH

Glucose and glycerol analyses were carried out enzymatically using glucose oxidase and glycerokinase respectively, employing commercial kits (glucose kit purchased from Sigma, UK and glycerol kit from Boehringer Mannheim, UK). Sufficient glucose and glycerol levels for analysis were provided by 200 μ l and 1000 μ l of centrifuged haemolymph respectively.

2.10 MEASUREMENT OF DISSOLVED INORGANIC CARBON (DIC) AND pH IN HAEMOLYMPH

Dissolved inorganic carbon levels were measured using a carbon dioxide gas-sensing electrode supplied by Orion, UK. The electrode was calibrated with standards prior to each use, checked hourly using a consistency standard and sample measurements corrected for any drift during the analysis period. The electrode was sensitive within the 10 to 1000ppm carbon dioxide concentration range (approximately 0.2 to 20mM). Three cm³ of sample were added to a glass tube containing a small magnetic stirring flea and 0.375 cm³ of citrate buffer (pH 4.5) and the mV response was monitored until it stabilised, a final reading being recorded at this point. Stabilisation typically took 5 minutes or so for each sample. All standards and samples were maintained and measured at the same temperature (27°C) using a water-bath. A total procedure blank, substituting deionised water for sample, gave negligible carbon dioxide at 0.075mM. Haemolymph samples have been analysed before and after freezing and there appears to be no difference in the values measured instantly or after thawing (D. Yellowlees pers comm.). Measurements of pH were taken using a Mettler pH meter calibrated with pH 7 and pH 9 buffers.

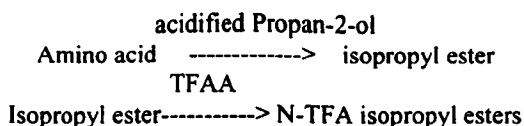
2.11 AMINO ACID ANALYSIS

2.11.1 Extraction and ion-exchange clean up of free amino acids for GCMS analysis.

Gloves were worn for all sample and standard manipulations for this technique, as amino acids from fingers are easily transferred to vessels (Walton and Curry 1991). One or two cm^3 of haemolymph were evaporated to dryness (or freeze dried) and subsequently extracted with 2-3 cm^3 of 80% ethanol. Centrifugation of the extracts from any residual material at 3500g for 5 mins was usually sufficient and the extracts were dried using centrifugal evaporation. The dried extracts were then cleaned up of any lipid or sugar components by cationic exchange using a scaled-up procedure of that by Adams (1974). A clean pasteur pipette was used as a column, using combusted quartz wool to keep 200 μl of Biorad AG50 X8 cationic resin (100-200 mesh) in place. The column was conditioned prior to use with 2 cm^3 of 1M hydrochloric acid followed by HPLC water until the eluant was pH 6-7, typically 4 cm^3 . The dried free amino acid residue was re-hydrated with 0.5 cm^3 HPLC water and 0.5 cm^3 pH adjusting solution (50% acetic acid in HPLC water). The stannous chloride (added to the pH adjusting solution in the method of Adams) which reduces cystine to cysteine, was omitted. The pH of the amino acid sample was checked at this point, the required pH being pH 2.0 - 2.5, adjustments were made with the acetic solution if necessary. The sample was added gently to the column and allowed to elute at a rate of 1drop/5s, followed by 1 cm^3 of HPLC water. Amino acids were eluted from the column with 2 cm^3 of 2M ammonia solution, increasing the flow rate with gentle air pressure to 1drop/s, into preweighed test tubes and dried under centrifugal evaporation. Dried extract weights were recorded and free amino acids were expressed as % free acids per dry weight of sample extracted.

2.11.2 Derivatization of amino acids into N-trifluoroacetyl isopropyl derivatives.

Amino acid derivatisation involves the esterification of the carbonyl group and the subsequent acylation of the amino group:



In this particular derivatization scheme, a modification of that used by Silfer *et. al* (1991), the esters are prepared by acid catalysed esterification using acidified propanol. As histidine and cysteine do not dissolve well in the higher alcohols, such as propanol, their analysis was omitted for this study. Although the problem can be circumvented by the formation of methyl esters first, which are then transesterified to the higher ester, this process adds yet another step where isotopic fractionation could occur and was avoided for any subsequent carbon isotopic analysis of amino acids.

The dried free amino acid extracts were mixed with 0.5 cm³ volumes of acidified propanol in 5cm³ reaction vials and incubated at 100°C for 30min (2,2-dimethoxypropane usually included as a water scavenger was omitted, as in acid solutions it reacts to form acetone and methanol. There is thus a possibility that some of the sample may esterify to methyl esters rather than complete conversion to isopropyl esters). The reaction was then quenched by placing the vials in the freezer for an hour, followed by evaporation to dryness under a gentle stream of nitrogen. After the addition of 0.25 cm³ of dichloromethane, the samples were evaporated under nitrogen. This last step was repeated once more. To the sample residue were added 0.5 cm³ of dichloromethane and 0.5 cm³ of TFAA, and after mixing, the samples were incubated at 100°C for 10min. On removal from the heating block the vials were plunged into an ice bath and 0.25 cm³ of dichloromethane added. The samples were then evaporated to dryness, with a nitrogen stream, over ice. The dry derivatives were stored at -20°C until analysis.

2.11.3 Analysis of TFA derivatives by GCMS.

Resultant N-TFA isopropyl esters were dissolved in approximately 50µl ethyl acetate (volume dependent on sample size) and analysed on a Hewlett-Packard 5890 GCMS, fitted with a BP5 column, film thickness 0.50µm, i.d.0.20mm, length 50 metres. The mode of injection was on column and Helium was used as the carrier gas. An initial oven

temperature of 100°C was held for 1 min, increased at a rate of 6°C/min until 200°C, followed by an increase to 250°C at a rate of 10°C/min and held at 250°C for 10 minutes. A solvent delay of 8 minutes was used. A mass scan range of 40 to 400 with a scan rate of 0.8scans/s was used. Identities of TFA isopropyl esters were made on the basis of comparison with retention times of known standards and by comparison of their mass spectra with library spectra using a NIST/EPA/MSDC database. Quantitation was performed using Hewlett-Packard Chemstation software, with TFA isopropyl esters being reported as per cent free amino acids.

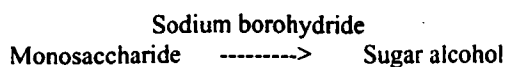
2.12 FREE MONOSACCHARIDES - EXTRACTION FROM HAEMOLYMPH

One to two cm³ of haemolymph were purified from any polar material using solid phase extraction cartridges using 100mg C18 solid phase columns (Jones Chromatography, UK), preconditioned with 2cm³ of methanol followed by 2cm³ of water Whiton *et al.* (1985). The haemolymph was added to the column and the eluant immediately collected into a pre weighed clean test tube. Another 1 cm³ of water was added to the column and collected also. The mono- and oligo-saccharide eluants were dried overnight in a vacuum oven at 30°C and stored at -20°C until derivatization.

2.12.1 Derivatisation of monosaccharides to produce alditol acetates.

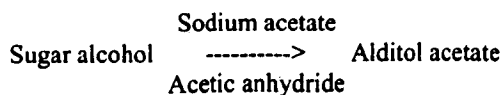
Monosaccharides require derivatization to produce volatile compounds to be analysed by GCMS and IRM-GCMS. Although it is possible to analyse monosaccharides without derivatization the elution time, with most conventional GC columns, proves to be prohibitively long. The following derivatisation procedure is a modification of that used by Fox *et al.* (1983) and Whiton *et al.* (1985) and was found to be the best technique for monosaccharide analysis since only one derivative is formed from each monosaccharide. The conversion of a monosaccharide into alditol acetate involves two steps. The first step in the derivatisation involves the reduction of each monosaccharide to its respective sugar alcohol. The second step is the acetylation of the alcohol to produce an acetate.

Step 1:



The above represents the reduction of the monosaccharide to its sugar alcohol, in the case of glucose this produces glucitol.

Step 2:



The above reaction is that of acetylation, using an excess of sodium acetate to catalyse the reaction.

Dried monosaccharide hydrolysates were dissolved in 250 μ l of water in a clean test tube, to which 100 μ l aqueous sodium borohydride solution (100mg/cm³) was added. After mixing, the solution was incubated at 4°C overnight. Borohydride reduced the monosaccharides to sugar alditols. Two cm³ of acetic acid/methanol (1/200) were then added to neutralise the borohydride reaction (and destroy excess borohydride), and the mix evaporated to dryness under vacuum at a temperature of 60°C. The acetic/methanol stage was repeated 4 times more, evolving sufficient sodium acetate to catalyse the next reaction. After the last evaporation the residue was dried for at least 3 hours before the addition of 600 μ l of acetic anhydride. Incubation at 100°C followed for at least 4 hours in 5 cm³ reaction vials. The vials were then cooled in the freezer along with a bottle of 80% v/v ammonium hydroxide. Preferably the vials were left for several hours before the addition, dropwise, of 0.8 cm³ of the ammonium hydroxide, over ice was usual, but not crucial if the vials were sufficiently chilled before addition. One cm³ of chloroform was then added to each vial and the vials shaken for several minutes, followed by centrifugation at 200g for 5 min to facilitate phase separation, into a top aqueous layer and lower chloroform layer containing the alditol acetates. The top aqueous layer was re-extracted with a further 2 cm³ of chloroform and the two chloroform layers combined. The chloroform extract was then washed of any polar material by the addition of 0.9 cm³ 1M HCl and mixed vigorously. After centrifugation the top aqueous layer was discarded and the lower chloroform layer,

transferred into preweighed vials, was evaporated to dryness using a low flow nitrogen stream. The alditol acetates were then dried overnight in a desiccator over anhydrous sodium sulphate. Several monosaccharide and sugar alcohol standards were also derivatized by the above method for use as identification aids. Blank sample work-up gave no detectable sugars on analysis.

2.12.2 GCMS of alditol acetates

Alditol acetates were dissolved in 50-500 μ l chloroform (dependant on sample size) and analysed on a Hewlett-Packard 5890 GCMS fitted with a SGE BPX-70 column, film thickness 0.25 μ m, i.d.0.20mm, and length 50 metres. The mode of injection was split (10:1) and Helium was used as the carrier gas. An initial oven temperature of 190°C, held for 1 min, was increased at a rate of 3°C/min until 260°C, which was held for 21 minutes. A solvent delay of 8 minutes was used. Spectra on the GCMS were acquired over the mass range 40-400 at a scan rate of 0.8 scan/s, using both total ion and selective ion monitoring. In selective ion monitoring mode, ion 115 was used for the detection of all neutral sugars, ion 84 was indicative of the amino sugars, glucosamine and galactosamine and ion 159 for deoxyribose. Selective ion monitoring is ideal for abundance measurements when other peaks are present, such as sugar fragments or reaction products where these extra peaks can essentially be ignored. Identities of alditol acetates were made on the basis of comparison with retention times of known standards and by comparison of their mass spectra with library spectra using a NIST/EPA/MSDC database. Quantitation was performed using Hewlett-Packard Chemstation software, with alditol acetates being reported as per cent alditol acetates. This does not directly translate into per cent free monosaccharides as several monosaccharides produce the same sugar alcohol on reduction with sodium borohydride. The 8 peak index was also used as an identification aid for sugars for which laboratory standards were not available.

3 TRANSLOCATION OF PHOTOSYNTHATES

3.1 INTRODUCTION

3.1.1 Aims for this section

1. To develop sample preparation techniques for the stable isotopic analysis of individual fatty acids isolated from *Tridacna* sp.
2. To identify and analyse the sources of fatty acids available to *Tridacna* sp. and to determine whether fatty acids are translocated to the host by the analysis of host and algal symbiont fatty acids.

3.1.2 Lipids and fatty acids

Lipids are important cellular constituents of all eukaryotic organisms, as the structural components of biological membranes, as energy sources and storage. Fatty acids are the simplest of lipids and have the general formula $R-COOH$, where R represents a hydrocarbon chain (Plate 4).

Free fatty acids occur only in trace quantities so they are generally found esterified as more complex lipids. In esters and other derivatives of fatty acids the $RC=O$ moiety, contributed by the acid, is called the acyl group. The storage lipids triacylglycerols, for instance, are composed of three fatty acyl residues esterified to glycerol (Plates 5 and 6). Stored in this form, fatty acids represent important metabolic fuels as the oxidation of fatty acids yields more energy per gram than the oxidation of carbohydrates, at 37kJ/g and 16kJ/g respectively. Triacylglycerols constitute the major lipid stores in both animals and plants and due to their hydrophobic nature they can be stored in an anhydrous form, unlike the storage of carbohydrates (e.g., glycogen) which requires water. Consequently triacylglycerols take up less space and mass within an organism and provide a better metabolic energy return per gram weight of stored biochemical component. Triacylglycerols are often quite diverse in their composition and depending on the fatty acid content can be either solids or liquids (oils) at

room temperature. Waxes, fatty acids esterified to alcohols (general formula; RCOOR), form protective and waterproof layers in plants and animals and are also major energy sources, especially in marine animals. Animals who live under the threat of starvation usually maintain a store of wax in addition to their usual energy supplies of triacylglycerols, proteins and glycogen.

More than 100 different fatty acids have been identified in micro-organisms, plants and animals and the development of techniques such as thin layer and gas chromatography since 1950 has allowed the extensive analysis of many plant species and algae. From these analyses it was found that fatty acids differ in several main features; the length of their hydrocarbon chain, the number of double bonds they may possess and the position of these bonds within the carbon chain. Common fatty acids of plant and animal origin have 10 to 24 carbon atoms and are generally even straight carbon chains though odd carbon chains do exist. So called saturated fatty acids are those with no double bonds whereas those with double bonds are referred to as unsaturated and may have up to six double bonds. Acids possessing several double bonds are called polyunsaturated acids (PUFAs). The configuration of the double bonds in unsaturated acids is generally *cis* with the bonds separated by a methylene group. Long chain fatty acids are prominent components of both the polar lipids of biomembranes and the triacylglycerols of cells and micro organisms (Erwin 1973). Environmental factors greatly influence the fatty acid composition of an organism, as does their nutritional state.

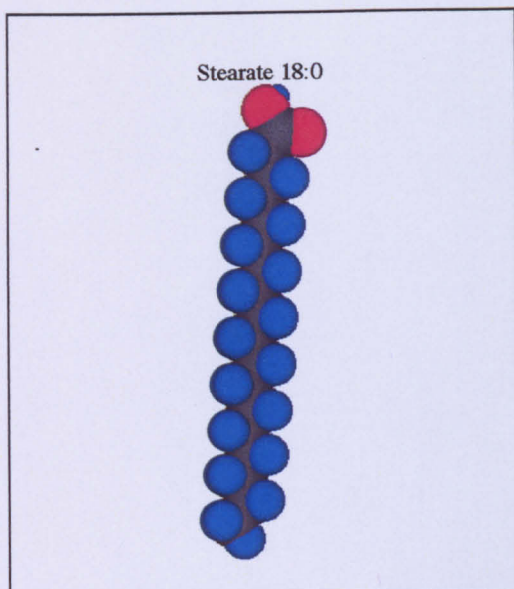


Plate 4.
A space filled molecular model of the saturated fatty acid stearate ($\text{CH}_3(\text{CH}_2)_{16}\text{CO}_2\text{H}$, octadecanoic acid). Carbon atoms are shown in black, oxygen in red and hydrogen in blue.

Plate 5
A ball and stick model of the three carbon alcohol, glycerol. Carbon atoms are shown in black, oxygen in red and hydrogen in blue.

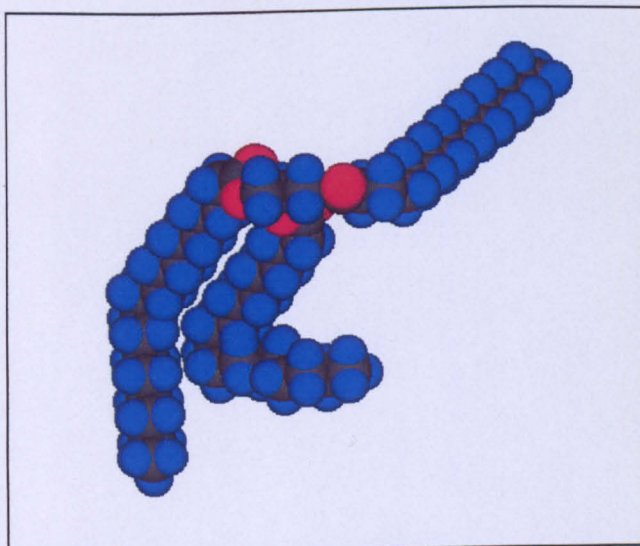
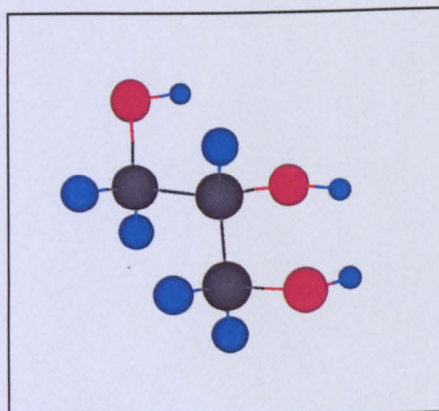


Plate 6
A space filled molecular model of the triacylglycerol Tristearin. Carbon atoms are shown in black, oxygen in red and hydrogen in blue.

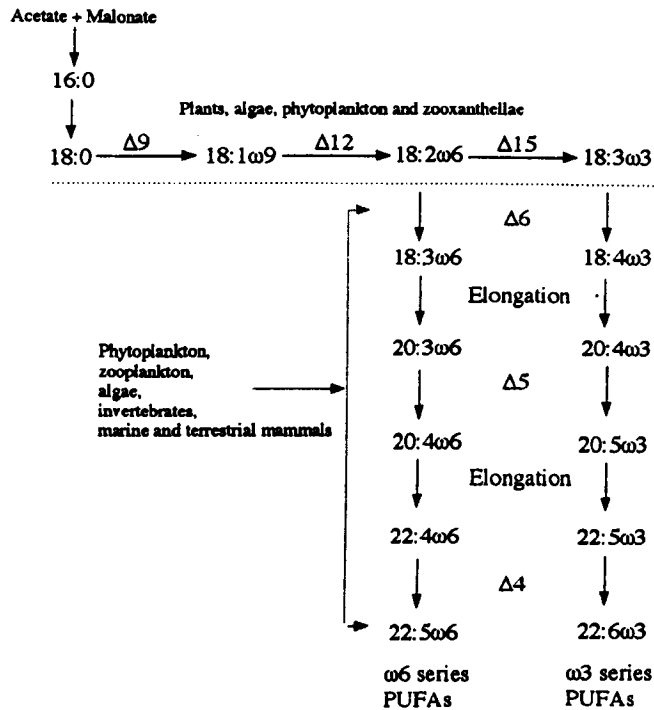


Fig. 3.1 Fatty acid synthesis, desaturation and elongation systems in the biosynthesis of essential fatty acids

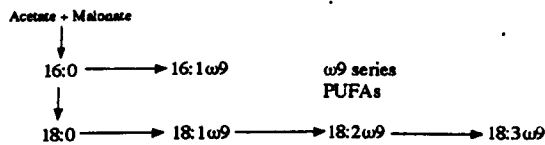


Fig. 3.2a *De novo* fatty acid synthesis in animals

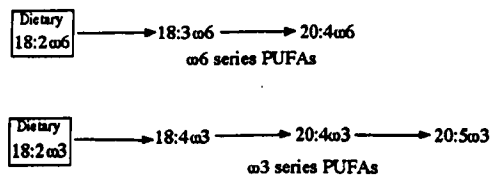


Fig. 3.2b Pathways of PUFA synthesis from dietary lipids

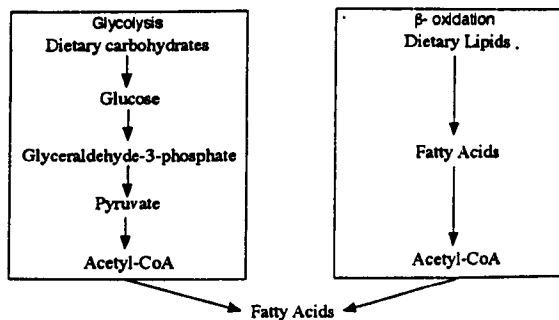


Fig. 3.3 Use of catabolic products to synthesise lipids by the clam

3.1.3 Distribution of fatty acids in plants, algae and animals

The fatty acids of animals are usually more simple in structure than those of plant origin. Plant fatty acids can be very complex because of the incorporation of several different functional groups such as epoxy-, hydroxy- and keto-groups, cyclopropane rings and acetylenic bonds. Palmitic acid is the major end-product of fatty acid synthesis in animal tissues and is the precursor for *de novo* synthesis of long chain saturated and unsaturated acids. Consequently the most abundant fatty acids found in animals are 16:0 (palmitic acid), 18:0 (stearic acid) and 18:1 ω 9 (oleic acid). Polyunsaturated fatty acids of animal origin are typically of the omega-6 series, 18:3 ω 6, 18:3 ω 9 and 18:3 ω 12 also 20:4 ω 6, 20:4 ω 9, 20:4 ω 12 and 20:4 ω 15. The acid 20:4 ω 6 is rarely found in plants. Plant acids are more typically of the omega-3 series such as 18:3 ω 3, 18:6 ω 3, 18:9 ω 3, 20:5 ω 3 20:5 ω 6, 20:5 ω 9, 20:5 ω 12 and 20:5 ω 15. Bacterial fatty acids are generally straight chain even numbered acids, although odd chain acids are more common in bacteria than in animals.

From the studies of plants and algae to date, several important patterns have emerged. There are differences in the fatty acid compositions of individual phyla of marine algae, in the compositions of marine versus freshwater algae and between algae and terrestrial plants. Triacylglycerols form the majority of dietary lipid that any animal will assimilate and the fatty acid composition of animal triacylglycerols is greatly influenced by their diet (Meyers 1979). If lipids are predominant in the diet, the synthesis of fatty acids from carbohydrates is suppressed in the tissues and the biosynthetic products of dietary lipid are synthesised directly into triacylglycerols. The implication of these findings is that it is possible to trace an animal's dietary lipids from analysing its tissue fatty acid composition.

3.1.4 Essential fatty acids

Polyunsaturated acids of the omega-3 and omega-6 series are thought to be essential dietary factors for marine (Bell *et al.*, 1986) and terrestrial animals (Gurr and Harwood, 1991). The polyunsaturated acids found in marine algae are synthesised via the serial desaturation and elongation of oleic acid (18:1 ω 9), (Fig.3.1) (Erwin, 1973; Gurr and Harwood, 1991). The enzymes in animals however, are only able to insert double bonds between the carbonyl group and the first double bond already present in the fatty acid whereas plant enzyme systems can only insert new double bonds between the last double bond and the terminal portion of the fatty acid. Hence animals can only desaturate between the Δ 9 position and the end of the carboxyl chain, whereas plants have the enzymes to desaturate at positions Δ 9,12 and Δ 15. *De novo* animal PUFAs are thus unlikely to have an omega number of less than 7 and are thus unable to make the acids 18:2 ω 6 (linoleic acid) and 18:3 ω 3 (linolenic acid) (Fig.3.1. and Fig.3.2.a). Yet these acids are considered essential to the animals growth, reproduction and good health, hence the term 'essential' fatty acids. Animals can, however, synthesise other polyunsaturated acids from the essential fatty acids by chain elongation and further desaturation (Fig.3.1 and 3.2b). It is generally believed that the desaturation and elongation steps tend to be inefficient so animals are unable to produce sufficient PUFAs for their needs and therefore have to source them from their diet. Plant oils are particularly abundant in linoleic and linolenic acids and fish oils are also abundant in linolenic acid. Linolenic acid is rarely a significant constituent of animal lipids suggesting that this particular acid is rapidly synthesised into another acid.

Arachidonic (20:4 Δ 5,8,11,14) and docosahexaenoic (22:6 Δ 4,7,10,13,16,19) acids (20:4 ω 6 and 22:6 ω 3) are derived from linoleic and linolenic acids respectively and are important as precursors of a group of compounds called eicosanoids. The eicosanoids have a variety of physiological effects at very low concentration, referred to as 'local hormones'; these compounds have an effect on almost every tissue of the body in animals.

Arachidonic acid is a major constituent of complex lipids in animals yet is rarely found in plants. Essential fatty acids (EFAs) are vital components of membranes where they are important regulators of membrane fluidity and play a role in membrane transport processes, EFAs also act as energy sources similar to saturated and monounsaturated acids (Guarnieri and Johnson 1970).

3.1.5 Importance of lipids for bivalve molluscs

Most marine animals utilise two forms of lipid storage, triacylglycerols and waxes, although the reason for this is unclear; it may be that the wax esters are more slowly metabolised than the triacylglycerols and thus provide a source of energy during times of starvation (Lee and Barnes, 1975). Previous studies have shown that triacylglycerols form the main lipid storage in zooxanthellae isolated from corals and anemones whilst waxes form only a small amount of storage lipid (Patton *et al.*, 1977; Blanquet *et al.*, 1979 and Harland *et al.*, 1991). There has only been one study of the lipid composition of zooxanthellae isolated from *Tridacna sp.* (*T. maxima*), in which galactosyl-diacylglycerols were found to be the major component of extracted lipids, forming 50% of the total (Bishop *et al.*, 1976).

Lipids and protein form the main energy reserves of marine bivalve larvae (Holland 1978) and aquaculture experiments have shown that certain PUFAs must be present in the diet of marine animals, especially during early growth stages at which time the animal may have limited capacity to synthesise particular fatty acids. The acids 20:5 ω 3 and 22:6 ω 3 were found to be essential for the nutrition of scallops and *N. atomus* (Whyte *et al.*, 1989; Volkman *et al.*, 1989). In contrast it has been shown that high proportions of saturated fatty acids can improve the growth of pacific oyster larvae (Thompson *et al.*, 1993). Lipids play a vital role in the reproductive success of *Tridacna* as gametogenesis is an extremely energy intensive process and requires the storage of lipid. Most marine animals, in contrast to terrestrial animals, do not have the ability to elongate polyunsaturated 18 carbon fatty acids into the acids 20:5 ω 3 and

22:6 ω 3, which are required for high growth rates and reproductive success. It is believed that the enzymes are present for these desaturations in some marine animals but their efficiencies are low and unable to meet the full requirements of the animal; this is particularly so at the time of gametogenesis. It is thus thought that both omega-3 and omega-6 series acids are essential dietary requirements for all marine animals (Bell *et al.*, 1986).

Phytoplankton and zooplankton are normally rich sources of the 20 and 22 carbon omega-3 fatty acids and are particularly rich in 20:5 ω 3 and 22:6 ω 3. Analysis of marine bivalves has shown that the acids 20:5 ω 3 and 22:6 ω 3 are predominant (Gardner and Riley, 1972; Paradis and Ackman, 1977 and Pollero *et al.*, 1979). All marine animals have high levels of these two acids as a proportion of their total polyunsaturated acids and it has been postulated that this feature may well represent a physiological adaptation to marine life (Malins and Wekell, 1969). It is thus reasonable to assume from these findings that all bivalves including the giant clams will also have essential fatty acid requirements (Gabbott, 1983).

3.2 SOURCES OF FATTY ACIDS AVAILABLE TO *TRIDACNA* SP.

To establish whether fatty acids are being translocated to the host in the *Tridacna* species it is important to determine all sources of lipid material available to the animal. The available sources can be broadly grouped into five categories.

3.2.1 Filter feeding

There are many sources of dietary organic carbon available for filter feeding bivalves, phytoplankton (micro, nano and pico), zooplankton, bacteria, larvae of various types, eggs released during spawning, dissolved organic material and other particulate organics such as detritus (dead organisms both animal and plant) and colloidal aggregates. These sources can be grouped under the heading particulate organic matter (POM). Living organisms form only a small fraction of the POM in sea water as a major proportion of POM is dead organic matter, so an important source of energy comes from bacteria (Finenko and Zaika 1970). The exact

composition of the POM changes daily and is dependent on the nutrient availability and temperature of the water column. The dietary intake of fatty acids is likely to be in the form of assimilated triacylglycerols and waxes as free fatty acids are unlikely to be a significant component. Dietary fatty acid composition is diverse and will include PUFAs. Acids may be used immediately by the clam unaltered or modified by elongation or desaturation provided the appropriate enzymes are present. All dietary sources are available to the Tridacnid clam, which is an efficient filter feeder. Although plankton are likely to be rich sources of fatty acids including the essential acids oligotrophic waters are not typically rich in plankton.

3.2.2 Dissolved organic matter (DOM)

Uptake of dissolved organic matter (DOM) may occur through the host tissues. The epidermis of the mantle in giant clams is highly convoluted (Norton *et al.* 1992) and well adapted for the active uptake of DOM (Fankboner, 1971). In the oligotrophic environment DOM is unlikely to be a significant contributor of lipids or fatty acids. Liberated fatty acids within the water column auto-oxidise rapidly, especially polyunsaturated acids, resulting in only saturated or mono-unsaturated acids at trace levels.

3.2.3 Translocated fatty acids from the zooxanthellae

The exact nature of lipid translocation is unknown in *Tridacna*. In a previous analysis of zooxanthellae (isolated from *T. maxima*) the zooxanthellae have been shown to possess large concentrations of 18:4 ω 3, 16:0, 18:5, 18:3 ω 6 and 22:6 ω 3. However, the analyses of dinoflagellates have shown them to be also rich in the PUFAs 20:5 ω 3 and 22:6 ω 3; fatty acids thought to be essential dietary requirements. Zooxanthellae may thus be a possible direct source of both saturated and polyunsaturated acids. The translocation of fatty acids from the zooxanthellae is thus a distinct possibility. Short chain fatty acids can dissolve in an aqueous medium and could be translocated in the clam haemolymph. In mammals lipids are transported in the blood stream attached to plasma albumin; however it is unknown whether

this mechanism exists in bivalve molluscs. There is evidence that the possession of zooxanthellae is important in clam development (Fitt and Trench, 1981; Fisher *et al.* , 1985; Fitt *et al.* , 1986). Clams do not survive beyond the veliger stage without their symbionts and it is possible the dependence could be related to essential fatty acids supplied by the zooxanthellae.

3.2.3 The synthesis of fatty acids *de novo* by the clam

Catabolic products of dietary intake may be used by the clam to synthesise lipids (Fig.3.3.). Lipid metabolism in bivalves has not been extensively studied although it has been demonstrated that several species of molluscs are capable of synthesising fatty acids from acetate (Voogt, 1983).

3.2.4 Synthesis of fatty acids using translocated algal glucose as the precursor

The clam may utilise translocated algal sugars to synthesise fatty acids *de novo* using acetyl CoA generated from glycolysis.

3.2.5 Terrestrial input

The runoff of organic material onto reefs can be a significant carbon source in some areas. One Tree Lagoon is not susceptible to this problem because of its distance from the mainland. It is unknown whether this is true for Orpheus Island, however, as part of the island is used for tourist facilities. It is unlikely that lipid material would present a significant source for benthic organisms from either area.

3.3 METHODS OF TRACING DIETARY SOURCES

3.3.1 Traditional methods

Classic techniques used to elucidate food webs and nutritional pathways involved the analysis of faecal matter or gut contents and time-consuming field studies for larger animals (e.g. Frafjord, 1993). Although the former techniques are useful they often only provide information

about dietary sources during the very recent past. In the case of a filter-feeding bivalve, analysis of faecal matter and gut contents may provide a snapshot view of the animal's diet for a single day, as indeed would the analysis of POM from the site. Analysis of the POM gives an indication of what may be available at any particular time but the bivalve may be selective in what is actually assimilated and some of the POM components may actively be rejected.

The introduction of radiocarbon tracers has greatly increased our knowledge of metabolic pathways and food web studies but experimental animals are often isolated from their normal surroundings and kept in laboratory conditions, consequently results may be biased accordingly. Radiotracers are also costly and laboratory personnel have to be trained in the use of radioactive compounds. Although radioactive tracers have been used to investigate the association in algal/invertebrate symbioses, it is often difficult to separate the symbiotic algae from their hosts especially when the algae are intracellular, such as in corals, to determine where the radioactivity has concentrated. Separation of zooxanthellar isolates from the host can also result in cell lysis of the algae possibly allowing radioactive tracers to leak from their original location.

3.3.2 Stable isotope methods in food web studies

DeNiro and Epstein (1976) suggested that for small animals an analysis of total animal carbon $\delta^{13}\text{C}$ provided an accurate measure of diet. However it is apparent today that whole body $\delta^{13}\text{C}$ is an inappropriate method for symbiotic animals since whole-body analysis would essentially be a mix of the two organisms and would not readily indicate the nutritional contribution to the host from the symbiotic algae. However in the last 20-30 years natural abundance stable isotopes have begun to replace classic ^{14}C radiotracer techniques in the elucidation of food webs and metabolic pathways. The analysis of an animal's tissue $\delta^{13}\text{C}$ provides information on metabolic processes that have resulted in the synthesised tissue and provides an integration of dietary carbon over a longer period than previous methods. Biochemical components in a tissue are constantly in a state of dynamic equilibrium with new components being synthesised and old components being degraded continuously (Bender 1975). Metabolically active tissues

such as fat tissue have a faster turnover rate than less metabolically active tissues such as connective tissue and bone in mammals. It is thus important to note that different tissues or biochemical fractions will have different turnover rates, so variability will be apparent in different organs both biochemically and isotopically. There are presently no data to suggest that the turnover rates in molluscs are similar to those in mammals but it is reasonable to assume that lipids will have a faster turnover rate, than structural protein for instance, in the bivalve *Tridacna*.

In any survey of biochemical components within a clam one would expect to see a mix of both normal dietary sources and symbiont sources. In non-symbiotic bivalves the lipid content of the animal is generally dependent on dietary lipid intake (Moreno *et al.* 1980; Piretti *et al.* 1987). Thus a method has to be utilised which will distinguish the two main carbon sources (dietary and symbiont derived) as simple abundance data may not accomplish this. For this I have chosen natural abundance carbon stable isotope analysis, primarily on biochemical components (compound specific isotope analysis). All biochemical fractions in an organism have a $\delta^{13}\text{C}$ signature that reflects the processes involved in the biosynthesis of that component. If a dietary component is utilised directly by the organism into a storage or structural product then the original isotopic signature of the component will be conserved. All subsequent enzymatic steps in the metabolism of an animal, and indeed plant, are likely to be accompanied by carbon isotopic fractionations. These fractionations are often predictable for particular metabolic pathways so the original signature is likely to be altered in a predictable way. Since clam fatty acids could be derived directly via translocation, indirectly as either elongated or desaturated algal acids, synthesised from other translocated material or from the catabolic products of filter feeding, in theory, it should be possible to determine which route from the $\delta^{13}\text{C}$ analyses. The analysis of $\delta^{13}\text{C}$ signatures in the clam may not positively define lipid pathways within the symbiotic association but may be able to support other available data.

The aim of this section of the study was to compare the natural abundances of fatty acids and their respective $\delta^{13}\text{C}$ isotopic signatures in *Tridacna* sp. with those same acids in non-symbiotic filter feeding bivalve contemporaries. From these data it was hoped that biochemical signatures could be identified in the symbiotic animals that were different from the non-symbiont animals. Due to the close relationship of the algae within the clam, zooxanthellae isolates were also prepared to identify zooxanthellar biochemical signatures that were compared to those of particulate organic matter (POM), taken again from the same site, which would represent the likely filter feeding diet for all animals. A survey of POM components was not taken, as only limited sampling was possible over a period of a week. In such sampling we are seeing only a snapshot view of any food available at that time, temporal sampling would undoubtedly reveal great variations in the composition of micro- and pico-phytoplankton and zooplankton within the water mass and also biochemical components.

Due to time constraints, the unique nature of these animals and also the fact that they are now included in the endangered species list it would have been inappropriate to undertake large-scale collections. Hence this study is not an extensive survey of $\delta^{13}\text{C}$ signatures in biochemical fractions within the Tridacnid symbioses, but serves to provide an insight into the possibility of using carbon isotopes to elucidate dietary versus symbiotic sources in a very complex relationship using a powerful new technique.

3.3.3 Using fatty acids to elucidate dietary sources

The analysis of fatty acid compositions of animals and potential food sources has been used previously to elucidate food sources in marine ecosystems (Jeffries, 1972; Schultz and Quinn, 1973). Conway and McDowell Capuzzo (1991) used fatty acid compositions and $\delta^{13}\text{C}$ of the total pooled acids to trace lipid biomarkers of bacterial endosymbionts of the bivalve *Solemya velum*. This is the first attempt at tracing photosymbiosis, using compound specific isotope analysis, in an association where algae are both the symbionts and are also the likely dietary

intake and where both symbiotic algae and phytoplankton are thought to utilise C3 photosynthesis pathways.

3.3.4 Existing evidence for translocation of lipids in algal/invertebrate symbioses

There is much evidence for translocation of lipids from zooxanthellae in several invertebrate/algal symbioses:

- (a) Fatty acids synthesised by the zooxanthellae, in the coral *Pocillipora*, are very similar to those stored in the host tissue lipids. It has also been speculated that coral fatty acid compositions are to a large extent controlled by algal biosynthesis and that much of the fatty acid content of the host is derived unaltered from the symbiotic algae (Patton *et al.*, 1977).
- (b) Several studies have reported the possible movement of lipids from zooxanthellae to the host in other invertebrate/algal symbioses (Muscatine and Cernichiar, 1969; Young *et al.*, 1971; Patton *et al.*, 1977 and Meyers *et al.*, 1978).
- (c) Several scientists have suggested the translocation of intact lipids from the zooxanthellae to the host (Crumeayrolles-Duclaux, 1969, Patton *et al.*, 1977, Blanquet *et al.*, 1979 and Crossland, 1980).
- (d) The presence of lipid droplets (composed of neutral lipids) in the cells of coelenterates has also been postulated as a possible mechanism of photosynthate transfer from the zooxanthellae to the host (Kellogg and Patton, 1983) and may represent the direct translocation of lipid. Controversy surrounds this particular finding as several studies have suggested that the presence of lipid droplets is due to sample preparation artefacts.
- (e) There is strong evidence that lipid synthesis in zooxanthellae is closely linked to photosynthesis (Patton *et al.*, 1977). During radiotracer studies, the zooxanthellae of the anemone, *Condyladis gigantea*, were found to incorporate very little radioactivity in the dark. However, in conditions of high irradiance, rapid lipid synthesis occurred with free fatty acids acquiring the highest specific activity, despite the fact that they comprised only a small proportion (<4%) of the total lipids in the algae (Kellogg and Patton, 1983). As the incubation time increased triacylglycerols and phospholipids gained higher specific activities suggesting

that the free acids were being incorporated into more complex lipids. Kellogg and Patton (1983) reported that it was impossible from the radiotracer studies alone to distinguish whether the lipid was being directly translocated to the host, or whether the lipid was being manufactured by the host, from some unknown soluble translocated photosynthate. Kellogg and Patton (1983) also demonstrated that the fat droplets were the main source of saturated fatty acids in the form of triacylglycerols, wax esters and free fatty acids and that PUFA were probably indicative of filter feeding sources.

(f) Zooxanthellae are able to synthesise fatty acids from ^{14}C -labelled acetate in *in vitro* studies (Blanquet *et al.*, 1979; Patton and Burris, 1983).

3.4 SAMPLE DETAILS

Total lipids and free fatty acids were extracted from Tridacnid tissues and respective isolated zooxanthellae, in conjunction with tissues from non-symbiotic filter feeding bivalve molluscs taken from the same sites at One Tree Island (species listed in Table 2.1, chapter 2). This strategy served to demonstrate any differences between the fatty acid profiles of the symbiotic versus the non-symbiotic animals that could be attributed to the symbiotic algae. *T. gigas* from Orpheus Island were also collected including a partial albino individual. Zooxanthellae were also isolated from five *T. gigas* from Orpheus Island to be used for the culture experiments detailed in section 3.6.6). Tables in the Appendices indicate those symbiotic and non-symbiotic animal tissues which were analysed for total lipids, free fatty acid profiles, bulk tissue $\delta^{13}\text{C}$ and carbon isotopic analysis of individual fatty acids. In order to determine whether there was any significant intraspecies variation in lipid content or fatty acid profiles the whole body tissues of several members of some species were analysed, notably a symbiotic species (*T. maxima* - 12 animals) and a non symbiotic species (*S. cucullata* - 29 animals). As mentioned previously, legislation exists, in the form of CITES, which prevents over sampling of Tridacnid species for research purposes and it was necessary to evaluate intraspecies variability both for fatty acids abundances and isotopic signatures in order to

justify the small sample numbers of individual tissues studied. The technique for isotopic analysis also was limited in the number of samples that could be processed, each individual fatty acid isotopic value requiring several analytical runs to provide statistical averages and standard deviations. The 12 *T. maxima* were already sacrificed from a previous experiment and were not collected solely for this project. Typical organs excised comprised of mantle, adductor muscle, gills, digestive mass and the remaining tissues were combined (remaining combined tissues, RCT). Several animals were too small for dissection and were analysed as whole body tissues. No attempt was made to isolate host tissue free of zooxanthellae in those tissues where algae were present in high density, however the adductor muscle had the outer epithelial layer removed which harbours many zooxanthellae. The zooxanthellar concentration in mantle is estimated to be less than 10%.

3.5 SOURCES OF FATTY ACIDS I: POM

Two possible sources of fatty acids for *Tridacna* sp., as described in section 3.2, were considered in this study: POM and zooxanthellar fatty acids. This section deals with POM results. Results for zooxanthellar acids are presented in section 3.6.

3.5.1 POM abundances and isotope composition

One Tree Lagoon

Particulate organic matter was collected from ten micro-atoll locations (lab names micro 1 to 10) in One Tree Lagoon, these locations were the identical locations for ten *T. maxima* collected for the study which were given the lab names Encore 1-12.

Concentrations of POM collected from the 10 locations at One Tree Lagoon ranged from 3.5-7.6mg l⁻¹ seawater collected (Table 3.1), assuming 0.67mg of organic matter is equivalent to 0.32mg of carbon (McAlistair *et al.*, 1960) this equates to 1672-3630 µg C l⁻¹. The concentration of POM at various sites in the Pacific ocean is very variable and according to Gordon (1971) ranges from 0.06 to 1250 µg C l⁻¹. McAlistair *et al.* (1960) also estimated 100-

150 $\mu\text{g C l}^{-1}$ in oligotrophic waters so the POM content at OTI lagoon appears to be quite high. The samples had from 0.2-2.4% lipid per gram dry weight of POM extracted (Table 3.1). Phyto- and zooplankters have been reported to contain 1.8-18.0% and 7.1-16.2% lipid respectively (Parsons *et al.* 1961; Serrazanetti *et al.* 1994). The low lipid content reported from POM in this study might reflect the paucity of plankton in this area and reflect a POM that is predominantly detrital in nature.

Orpheus Island, Pioneer Bay

Two samplings collected at Pioneer Bay had POM concentrations of 40.7 and 85.6 mg l^{-1} seawater collected. These values were some ten times those at One Tree Lagoon and apparently reflect the less oligotrophic nature of the waters at Orpheus compared to One Tree Lagoon. Concentration of lipid in the POM however was similar to One Tree Lagoon at 1.3% per gram dry weight of POM extracted (Table 3.1).

Table 3.1. Particulate organic matter (POM) collected from ten locations at One Tree Reef, the JCU aquarium and two locations in Pioneer Bay, Orpheus Is. POM calculated as mg l⁻¹ of filtered seawater, total lipid as % dry weight of material extracted.

Location	POM mg l ⁻¹	Total lipid % dry weight
One Tree Is		
Micro atoll 1	6.3	0.2
Micro atoll 4	7.6	0.3
Micro atoll 6	4.4	0.5
Micro atoll 7	4.6	0.5
Micro atoll 8	6.2	1.2
Micro atoll 9	3.5	0.5
Micro atoll 10	6.5	2.2
Micro atoll 11	5.2	2.4
Micro atoll 12	6.7	0.8
Mid lagoon	3.6	1.8
Average	5.5 ± 1.4	1.0 ± 0.8
Townsville		
JCU aquarium	227.7	4.3
Orpheus Is.		
Pioneer Bay	85.6	1.3
Pioneer Bay	40.7	
Average	63.2	

JCU aquarium

POM levels were considerably higher in the aquarium at 227.7mg l⁻¹ seawater collected, as was the level of lipid at 4.3% compared to One Tree Lagoon and Pioneer Bay (Table 3.1).

Table 3.2. $\delta^{13}\text{C}$ isotopic data for phytoplankton, zooplankton, POM and DOC at various locations around the world. Isotopic data are expressed as $\delta^{13}\text{C}_{\text{PDB}} (\text{‰})$.

Location	Sample type	$\delta^{13}\text{C}_{\text{PDB}} (\text{‰})$	Source
Off coast of Peru and Ecuador	Phytoplankton	-19.3	Degens <i>et al.</i> 1968b
Bering Sea, NW Pacific	Phytoplankton	-24.4	McConnaughey 1979
Bering Sea, NW Pacific	Zooplankton	-22.1	McConnaughey 1979
Off coast of Southern California	Zooplankton	-18.6, 19.7	Williams and Gordon 1970
Discovery Bay, Jamaica	Phytoplankton	-17.9	Land <i>et al.</i> 1975
Discovery Bay, Jamaica	Zooplankton	-17.6 to -17.1	Land <i>et al.</i> 1975
Southern Benguela, South Africa	Phytoplankton	-20.5 to -19.8	Monteiro <i>et al.</i> 1991
Southern Benguela, South Africa	Zooplankton	-18.5 to -15.5	Monteiro <i>et al.</i> 1991
Tampa Bay, Florida	Phytoplankton	-21.3 to -20.1	Conkright and Sackett 1986
Tampa Bay, Florida	POM	-23.2 to -19.6	Conkright and Sackett 1986
West coast of France	POM	-21.8 to -19.5	Riera and Richard 1996
Temperate region compilation	POM	-23.8 to -19.3	Gearing <i>et al.</i> 1984
Semi tropical, Gulf Mexico, Caribbean, Atlantic	POM	-22.3 to -19.8	Eadie and Jeffrey 1973
Gulf of Mexico	POM	-22.2	Calder and Parker 1968
Gulf of Mexico	DOC	-21.6	Calder and Parker 1968
Polar, South Indian Ocean	POM	-26.0 to -24.7	Eadie and Jeffrey 1973
Northeast Pacific	POM	-24.4 to -21.2	Williams and Gordon 1970
Northeast Pacific	DOC	-24.3 to -22.0	Williams and Gordon 1970

Isotopic composition of bulk POM

Previous $\delta^{13}\text{C}$ values for POM sampled from various sites around the world in seven studies ranged from -26.0 to -19.3‰, with a similar range for phytoplankton, zooplankton and dissolved organic matter (DOC), Table 3.2. Bulk carbon isotopic data were not obtained for this study but it is assumed that isotopic compositions would be within the range above. Carbon isotopic data for individual fatty acids isolated from POM are given in section 3.5.3.

The bulk composition is very much determined by the biochemical components within the POM, and for live phytoplankton and zooplankton, it is a reflection of the organism's nutritional state at that particular time.

3.5.2 Free fatty acids isolated from POM

One Tree Lagoon

The fatty acid profiles for POM taken at ten different locations from One Tree Lagoon show slight variability in their most predominant fatty acids. This most likely reflects the dominant algal species present at each site (Fig.3.4) The carbon isotopic data for individual fatty acids in this plot and in the following plots are positioned next to the relevant fatty acid. Any bulk tissue isotopic data in following plots are positioned either within or above the fatty acid abundance profile. Phytoplankton species present were not determined for this study.

Saturated acids, 14:0, 16:0 and 18:0 occur in all of the ten POM samples, comprising 2.6-16.2%, 11.7-30.8% and 5.3-15.0% of the total acids respectively. The odd chain 15:0 occurred in only three of the ten sites (Micro atolls 1, 12 and 7) and at concentrations of less than 2.5%.

Monoenoic acids formed between 12.6-52.6% of the total acids in the ten sites. The acids 16:1 ω 9 and 18:1 ω 7, were both less abundant than their 16:1 ω 7 and 18:1 ω 9 isomers, and each formed less than 5% of the total acids. The major monoenoic acid, 18:1 ω 9, comprised 7.4-26.5% of the total and 16:1 ω 7, 2.6-8.6%. The acid 20:1 occurred in eight of the ten samples but at concentrations of less than 5.5%.

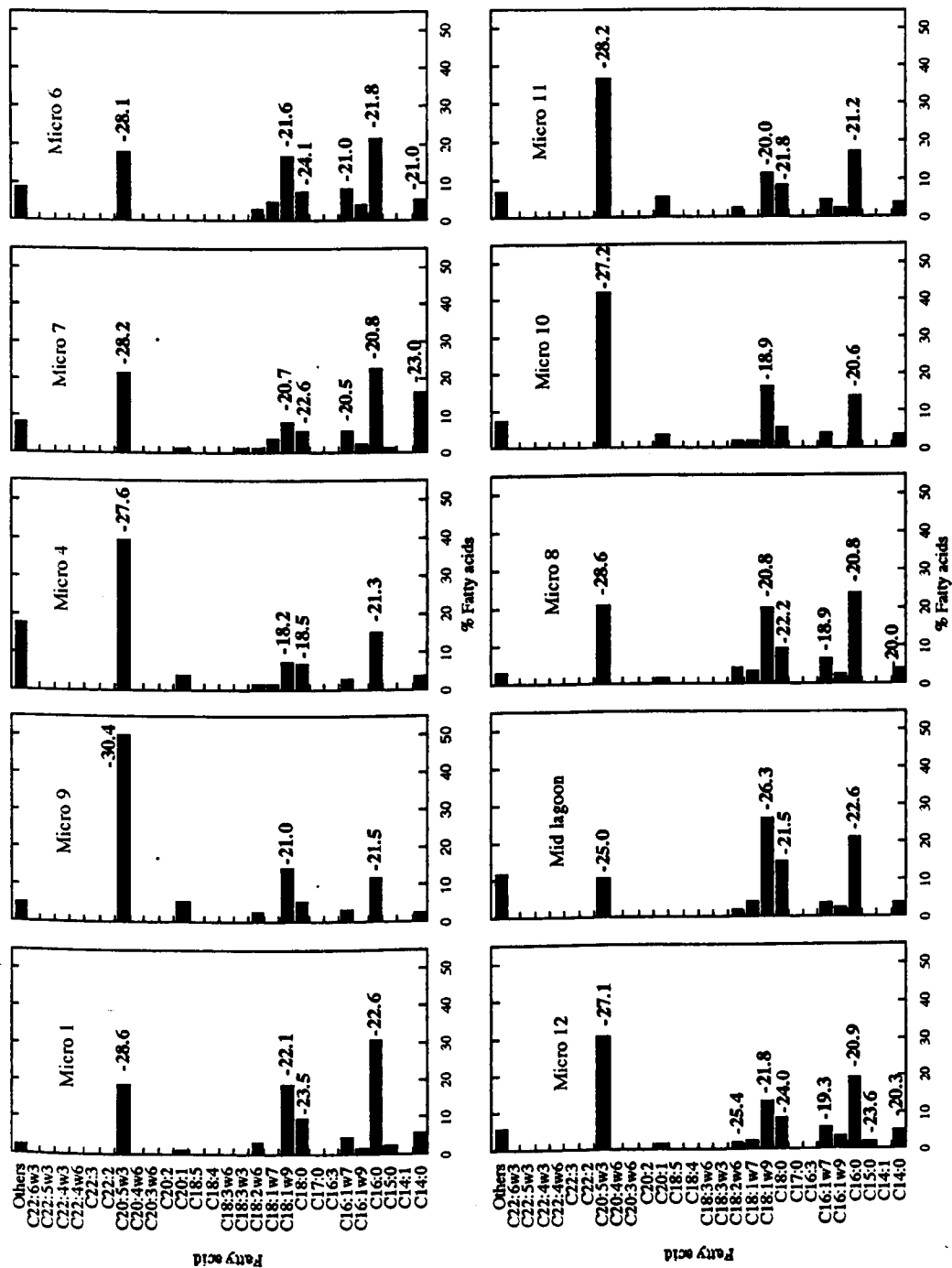


Fig. 3.4 Fatty acid and isotopic profiles for particulate organic matter (POM) collected from ten sites in One Tree Lagoon. Carbon isotopic compositions (δ¹³C ‰) of individual fatty acids are placed next to the relevant fatty acid.

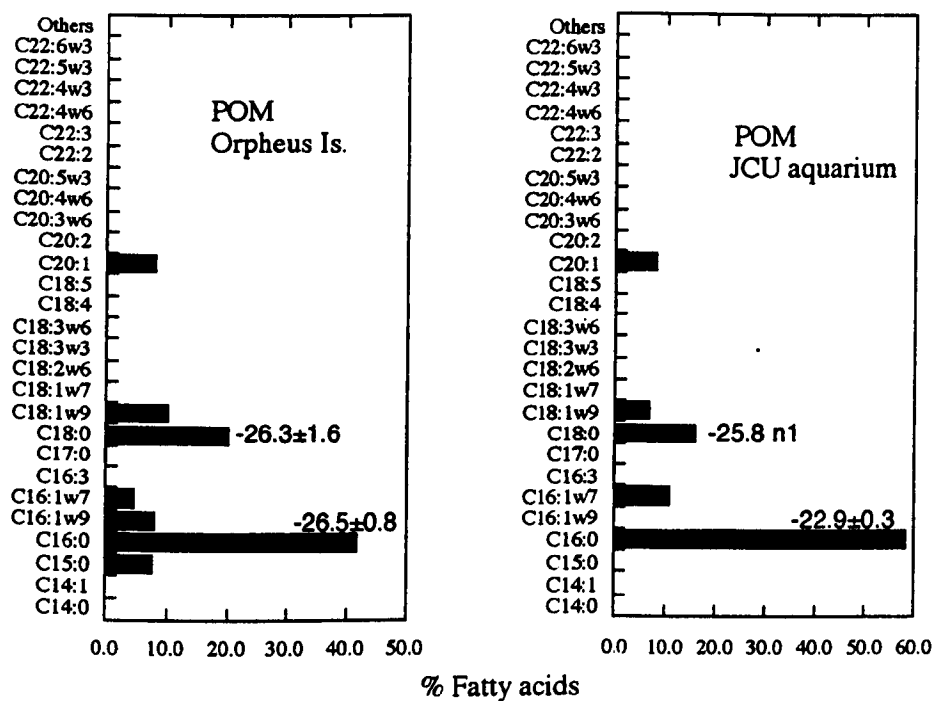


Fig. 3.5 Fatty acid profiles of particulate organic carbon (POM) collected from Pioneer Bay, Orpheus Is. and the James Cook University aquarium. Carbon isotopic data ($\delta^{13}\text{C}$ ‰) for individual fatty acids are given next to relevant fatty acid.

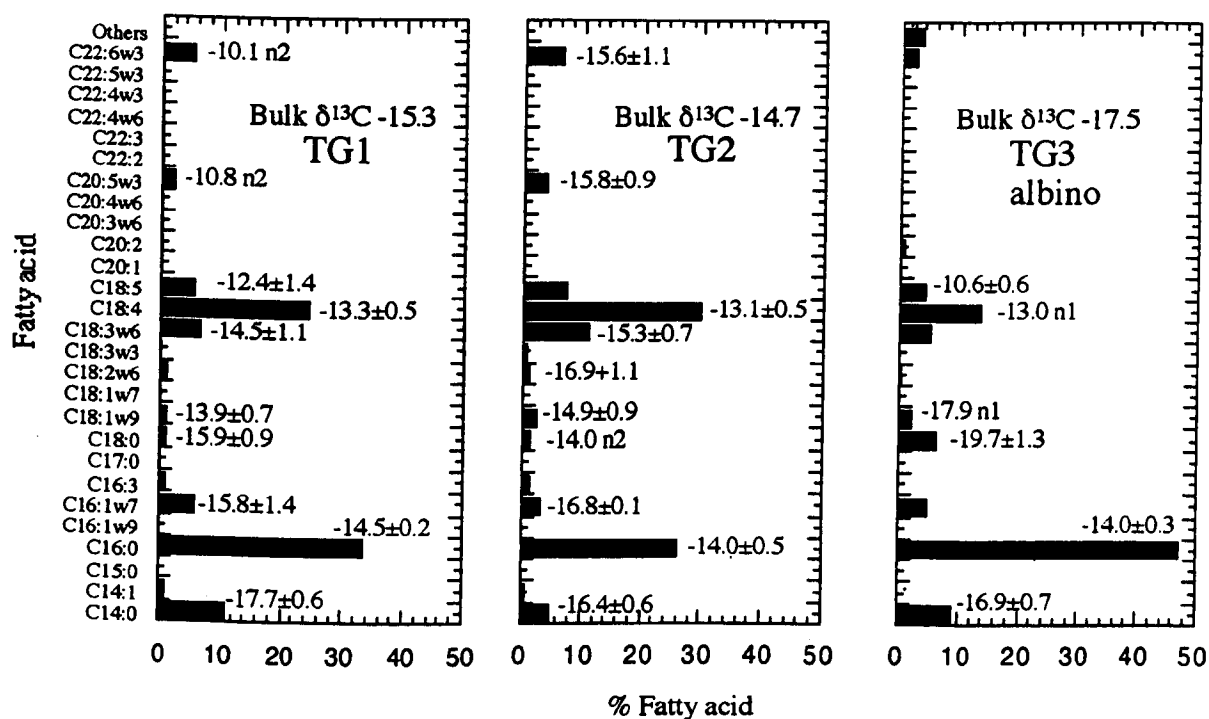


Fig. 3.6 Fatty acid and isotopic profiles of zooxanthellae isolates from the symbiotic clam *T. gigas*. Animals TG1 and the partial albino TG3 were collected directly from Pioneer Bay, Orpheus Is. Animal TG2 (originally from Pioneer Bay) was held in the JCU aquarium for several weeks before sacrificing.

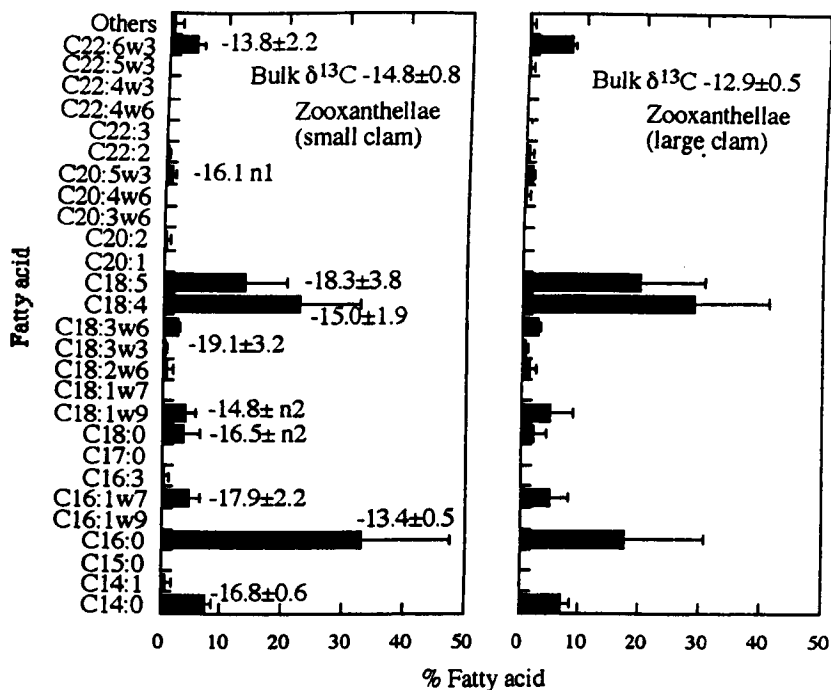


Fig. 3.7 Fatty acid and isotopic profiles of zooxanthellae isolates from the symbiotic clam *T. maxima*. Fatty acid profiles and bulk $\delta^{13}\text{C}$ values (‰) represent average values for 4 individuals and 5 individuals for small and large clams respectively. Individual fatty acid $\delta^{13}\text{C}$ isotopic data are from one animal (TM1), the isotopic data represent an average ($\pm 1\text{s}$) for multiple analysis ($n=5$) of that sample

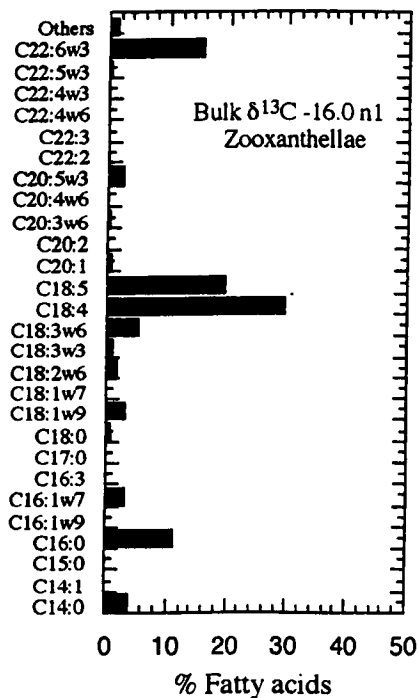


Fig. 3.8 Fatty acid profile for zooxanthellae isolated from the symbiotic bivalve *T. squamosa*. Fatty acid abundances are based on the single analysis of one individual

Polyunsaturated acids formed 12.6-52.6% of the total acids in POM. Only two polyunsaturated acids were consistently found in all of the ten sites, the acids 18:2 ω 6 and 20:5 ω 3. By far the most predominant was 20:5 ω 3, present in concentrations between 10.5-49.9%. 18:3 ω 3 was present at a concentration of 1.2% in Micro atoll 7 but absent from all other samples. Unsaturation indices for 9 of the samples are in the range 128-278 and reflect the high degree of unsaturation for the fatty acids present in POM. (The unsaturation index is calculated by adding together; 1 x the abundance of saturated acids + 2 x the dienoic acids + 3 x the trienoic acids and so-on for all the PUFAs). The mid-lagoon sample however has a lower index of 93, this difference is entirely due to the lower concentration of 20:5 ω 3 in this particular sample compared to other samples. Omega 3 acids formed between 10.5-49.9% (all 20:5 ω 3) and omega 6 acids 1.4-4.5%. The average ω 6/ ω 3 ratio for POM at the ten sites was low and averaged 0.1 (Data table in appendices). This confirms previous reports that plant and algal fatty acids are predominantly of the omega 3 series (Pohl and Zurheide, 1979; Sargent *et al.*, 1987).

The ten POM sites fall roughly into 4 groups based on their fatty acid composition.

(1) Micro atolls 1 and 8 have similar profiles with 16:0, 20:5 ω 3, 18:1 ω 9 and 18:0 forming the most predominant acids (in order of abundance) these acids form 78.3% and 73.9% of the total fatty acids in micro atoll 1 and 8 respectively. (2) Micro atolls 4, 11, 12 and mid lagoon all have the fatty acids 20:5 ω 3, 16:0, 18:1 ω 9 and 18:0 as the most abundant, these four acids comprising 69.1, 73.7, 71.5 and 73.2% of the total respectively. (3) Micro atolls 9 and 10 have very similar profiles and their three most predominant acids are both 20:5 ω 3, 18:1 ω 9 and 16:0, these acids comprising 72.0 and 65.1% of the total. They differ only in their 4th most abundant acid, Micro atoll 9 possess 20:1 whilst Micro atoll 10 has 18:0. (4) Micro atolls 6 and 7 both possess 16:0 and 20:5 ω 3 as their two most abundant acids but differ in their 3rd and 4th most abundant acids. In Micro atoll 6 these acids are 18:1 ω 9 and 16:1 ω 7 whereas in Micro atoll 7 they are 14:0 and 18:1 ω 9 (in order of abundance).

The acids 20:5 ω 3, 16:0, 18:1 ω 9, 18:0, 16:1 ω 7, 14:0 form over 60% of all the fatty acids extracted from POM from One Tree lagoon. The acid 20:5 ω 3 in particular was present in large amounts in all the samples. Table 3.3 shows POM from One Tree Lagoon, average values for the ten collection sites in Fig.3.4 and will be used in section 3.8 to infer nutritional sources for *T. maxima*.

Table 3.3. Average fatty acid abundances and average isotopic values of particulate organic matter (POM), collected from One Tree Lagoon. Values represent average values for 10 sites. Error bars = $\pm 1\sigma$ on both fatty acid % and on $\delta^{13}\text{C}$ values. Isotopic values expressed as $\delta^{13}\text{C}_{\text{PDB}}$ (‰).

Fatty acid	% total fatty acids	$\delta^{13}\text{C}_{\text{PDB}}$ (‰)	Fatty acid	% total fatty acids	$\delta^{13}\text{C}_{\text{PDB}}$ (‰)
C14:0	5.4 \pm 3.9	-21.1 \pm 1.3	C18:5	0	-
C14:1	0	-	C20:1	2.4 \pm 2.0	-
C15:0	0.6 \pm 1.0	-23.6 (n=1)	C20:2	0	-
C16:0	19.7 \pm 5.6	-21.4 \pm 0.7	C20:3 ω 6	0	-
C16:1 ω 9	2.0 \pm 1.5	nd	C20:4 ω 6	0	-
C16:1 ω 7	5.0 \pm 1.8	-19.9 \pm 1.0	C20:5 ω 3	28.8 \pm 12.7	-27.9 \pm 1.4
C16:3	0	-	C22:2	0	-
C17:0	0	-	C22:3	0	-
C18:0	8.2 \pm 2.8	-22.3 \pm 1.8	C22:4 ω 6	0	-
C18:1 ω 9	15.3 \pm 5.7	-21.1 \pm 2.2	C22:4 ω 3	0	-
C18:1 ω 7	2.2 \pm 1.8	nd	C22:5 ω 3	0	-
C18:2 ω 6	2.5 \pm 0.9	-25.4 (n=1)	C22:6 ω 3	0	-
C18:3 ω 3	0.1 \pm 0.4	-	Others	7.7 \pm 4.4	nd
C18:3 ω 6	0	-	Total	100	
C18:4	0	-			

This particular suite of acids are characteristic of several marine phytoplankters such as Chrysophyceae, Xanthophyceae, Eustigmatophyceae and Bacillariophyceae (Pohl and Zurheide, 1979; Volkman *et al.*, 1993). The acids 16:0, 16:1 and 20:5 typically comprise more than 50% of the total fatty acids in most diatoms and a low content of 18 carbon fatty acids is characteristic for this class (Pohl and Zurheide, 1979). The largest proportion of fatty acids in most Dinophyceae is made up of 16:0, 18:4, 20:5 ω 3 and 22:6 ω 3, and it is of note that the acid 22:6 ω 3 was absent in all POM samples collected. Phaeophyceae are also reported to

have high levels of 18 and 20 carbon acids, especially the acids 18:3, 18:4, 20:4 and 20:5, although neither 18:4 nor 20:4 were found in any of the samples and 18:3 occurred only in Micro atoll 7 at a very low abundance (circa 1%). Red algae (Rhodophyceae) are particularly noted for the formation of high amounts of polyunsaturated 20 carbon acids especially 20:4 and 20:5, with 20:5 usually being the most abundant. Chlorophyceae are characterised by a wide variety of saturated and unsaturated acids but primarily synthesise 16:0 and 18:0. Cryptophyceae are characterised by the acids 16:0, 16:1, 18:3, 20:1 and 20:5. The most striking feature of this class is the high content of 20:1 which is unique among all other algae (Pohl and Zurheide, 1979). This acid occurs in all but two microatolls but in fairly low abundance 1.3-5.5%. At present there are no published data on the likely seasonal algal composition of One Tree Lagoon.

It is difficult to hypothesise on the actual composition of the dominant algal classes present in the water column at One Tree; it is clear that an abundance of the essential fatty acid 20:5 ω 3, would be available for immediate use by a filter feeding bivalve.

Pioneer Bay, Orpheus Island

Saturated acids formed 68.7% of the total in the POM collected from Pioneer Bay (Fig.3.5). The lack of unsaturated acids in this sample is reflected by the very low unsaturation index of 30. The acids, 15:0, 16:0 and 18:0 comprising 7.7%, 41.7% and 20.3% of the total acids respectively.

Monoenoic acids formed 30.3% of the total acids in this sample. 18:1 ω 9, comprised 10.1%, 16:1 ω 9; 8.0% and 16:1 ω 7; 4.4%. The 16:1 ω 9/16:1 ω 7 ratio was 1.8. The acid 20:1 was also present and comprised 7.8% of the total.

Polyunsaturated acids were not found in this POM sample which may indicate possible auto-oxidation of the sample. Alternatively the fatty acid profile may simply reflect a POM which

is not primarily composed of phyto- and zooplankton and primarily detrital in origin. Data tables are given in appendices.

JCU aquarium

Saturated acids, 16:0 and 18:0 only, occur in the POM collected from the aquarium, comprising 41.7% and 20.3% of the total acids respectively. This sample also has a very low unsaturation index of 26 (Fig.3.5).

The monoenoic acids 16:1 ω 7, 18:1 ω 9 and 20:1 are the only other acids present, comprising 10.7, 6.8 and 8.1%. As in the previous sample PUFAs are totally absent. From the limited data it does appear that both the Orpheus and aquarium samples may have degraded in transit.

3.5.3 $\delta^{13}\text{C}$ of individual fatty acids extracted from POM

Studies by Sackett *et al.* (1965) and Degens *et al.* (1968a and 1968b) showed that there was no appreciable difference between the bulk $\delta^{13}\text{C}$ of zooplankton and phytoplankton living in the same environment. However, other published data in Table 3.2 suggest there may be small differences between zoo- and phytoplankton at the same site; between 0.3 and 5.0‰, for the data tabulated. The measurement of POM, however, gives a fair indication of the bulk $\delta^{13}\text{C}$ dietary composition available to filter feeding bivalves.

The $\delta^{13}\text{C}$ isotopic values of individual fatty acids, extracted from One Tree Lagoon POM, were very similar for each of the ten sites sampled and ranged from -18.2 to -30.4‰. This range is very similar to that of bulk $\delta^{13}\text{C}$ values reported for POM, phytoplankton and zooplankton (Table 3.2). Saturated and monoenoic acids were typically less ^{13}C -depleted than the more desaturated acids. The average $\delta^{13}\text{C}$ value for 20:5 ω 3 was around 6.5‰ more ^{13}C -depleted than for 16:0. This presumably reflects the fractionation exhibited by enzymes that elongate and desaturate fatty acid chains. The ^{13}C -depletion of PUFAs, relative to 16:0, in this study is larger than previously reported for acids produced from one short chain precursor (Schouten *et al.*, 1998). Average fatty acid abundances and isotopic signatures for POM from

OTI are given in (Table 3.3) and, within the limitations of such a statistically small sample population, represent an average POM for the site.

Due to the low abundance of unsaturated fatty acids in POM samples for Pioneer Bay and the JCU aquarium only the saturated acids 16:0 and 18:0 yielded isotopic data. $\delta^{13}\text{C}$ values ranged from -25.8 to -26.5‰ for the two fatty acids. As demonstrated earlier, in section 2.8.2, even if these samples have undergone auto-oxidation, the isotopic signatures for the remaining acids are likely to be representative of the fresh samples. The loss of PUFAs in these two samples prevents a full comparison the samples with POM from OTI, however, the saturated acids 16:0 and 18:0 from both Orpheus and JCU aquarium samples are typically 4‰ more ^{13}C -depleted than those same fatty acids from One Tree Lagoon.

3.6 SOURCES OF FATTY ACIDS II: ZOOXANTHELLAE

3.6.1 Total lipids of zooxanthellae

Zooxanthellae isolates had total lipid concentrations ranging from 12.6-52.5% in the species *T. gigas*, *T. maxima* and *T. squamosa*. The two species collected from One Tree Lagoon (*T. maxima* and *T. squamosa*) and the partial albino *T. gigas* (collected from Pioneer Bay, Orpheus Is,) had significantly lower zooxanthellar lipids than the two normal *T. gigas* animals from Pioneer Bay, Orpheus Is (Table 3.4).

Table 3.4. Total lipid content in zooxanthellae isolated from symbiotic bivalve mantle tissue. Lipids are expressed as percent lipids per dry weight of tissue extracted, values are average values when more than one individual was analysed. Numbers in parenthesis refer to the number of samples analysed. Lipid concentrations from previous zooxanthellae and dinoflagellate studies are included for comparison.

Species	Lipid content (%dry weight)	Source
Symbiotic algae:		
Zooxanthellae (<i>T. gigas</i>)	52.5 (2)	This study
Zooxanthellae (<i>T. gigas</i> - partial albino)	12.6 (1)	This study
Zooxanthellae (<i>T. maxima</i>)	21.1±14.8 (9)	This study
Zooxanthellae (<i>T. squamosa</i>)	13.8 (1)	This study
Average	25.0	
stdev	18.7	
Zooxanthellae (<i>Condylactis gigantea</i>)	38.9	Kellogg and Patten 1983
Zooxanthellae (<i>A. viridis</i>)	24.4	Harland <i>et al.</i> 1991
Average	31.7	
stdev	10.2	
Dinoflagellates:		
<i>Amphidinium carteri</i>	18	Parsons <i>et al.</i> 1961
<i>Exuviella</i> sp.	15	Parsons <i>et al.</i> 1961
<i>Glenodinium</i> sp.	22	Harrington <i>et al.</i> 1970
<i>Gyrodinium</i> sp.	14	Harrington <i>et al.</i> 1970
Average	17.2	
stdev	3.6	

High lipid contents (38.9±6.7% total lipids) in zooxanthellae have been reported previously in the cnidarian *Condylactis gigantea* (Kellogg and Patton, 1983). The average lipid content for zooxanthellae in this study (25.0±18.7%) agrees well with that found by Kellogg and Patton (1983) and Harland *et al.* (1991) in their analysis of zooxanthellae isolated from *Condylactis gigantea* and *Anemone viridis* respectively (Table 3.4).

3.6.2 Free fatty acids from zooxanthellae

Zooxanthellar fatty acid profiles for *T. gigas*, *T. maxima* and *T. squamosa* are given in Figs.3.6, 3.7 and 3.8 respectively. Saturated fatty acids formed 31.1 to 38.6%, 34.1±18.0% and

16% respectively in the species *T. gigas*, *T. maxima*. and *T. squamosa*. However, the partial albino specimen of *T. gigas* had a significantly higher saturated acid content at 62.8%. The average saturation index for this animal's zooxanthellae was 116 compared to 200 and 260 for the two unbleached animals. Unsaturation indices ranged from 88 to 374 in zooxanthellae isolated from *T. maxima* and 354 for *T. squamosa* zooxanthellae Data tables are given in appendices. No correlation was observed between shell size and unsaturation index in *T. maxima*.

Saturated acids

Only about half of the isolated zooxanthellae from *T. maxima* had 16:0 as the most predominant fatty acid (the other half of the isolates had 18:4 as the most abundant fatty acid and the data are described below), although this acid was always one of the four most abundant acids in all isolates and accounted for 25.6-54.3% of the total zooxanthellar acids isolated from *T. gigas*, *T. maxima* and *T. squamosa*. The fatty acids 14:0 and 18:0 were also present in all species, typically the 14:0 being more abundant. Odd chain saturated acids were not present as free acids of zooxanthellae.

Monounsaturated acids

Monoenoic acids 16:1 ω 7 and 18:1 ω 9 were present in all zooxanthellae analysed and comprised typically <10% of the total. Only one of nine individuals of the species *T. maxima* had 14:1 (2.3%) in its zooxanthellae isolates. 14:1 was also found in zooxanthellae from normal *T. gigas* (0.7-1.2%) but was absent in the partial albino animal.

Polyunsaturated acids

The most predominant PUFAs in zooxanthellae isolated from *T. gigas*, *T. maxima* and *T. squamosa* were 18:4, 18:5, 22:6 ω 3 and 18:3 ω 6. The acid 18:4 was frequently the most abundant of all acids and was present in the range 9.0 to 39.3%. Analysis of zooxanthellae

from small and large specimens of *T. maxima* revealed that 18:4 and 18:5 were the most predominant of all fatty acids in large animals but in small animals the saturated acid 16:0 dominated. The albino *T. gigas* also possessed lower values of 18:4 and 18:5 compared to their non-bleached contemporaries. The PUFA 18:4 is considered to be a fatty acid "marker", its presence indicative of flagellates and dinoflagellates (Kattner 1989). The acid 18:5 is usually found with 18:4 in phytoplankton and it is thought to derive from 20:5 by loss of acetate rather than from 18:4 by desaturation (Mayzaud *et al* 1976). Although present at low abundance the acids 18:2 ω 6 (0.5-3.0%) and 20:5 ω 3 (0.9-3.8%) were usually found in zooxanthellae. The ω 6/ ω 3 ratios of these zooxanthellae appear to be an indication of the filter feeding contribution to the host clam. *T. maxima* and *T. squamosa* zooxanthellae, whose hosts were collected from a more oligotrophic environment than Orpheus Island or the JCU aquarium, have lower ω 6/ ω 3 ratios (0.3 to 0.7) than *T. gigas* zooxanthellae (1.0 and 1.1), Tables 3.5, 3.6 and 3.7. The albino *T. gigas* zooxanthellae (2.3) possesses the highest ω 6/ ω 3 ratio. This could be an indication of the host's increased dependence on filter feeding for its nutrition compared to its fully zooxanthellate contemporaries and that some fatty acids are possibly recycled to the zooxanthellae. A recycling of host fatty acids hypothesis may also explain the distribution of fatty acids in small versus large *T. maxima*, in that the acids 18:4 and 18:5 may be recycled to the resident zooxanthellae.

Table 3.5 Fatty acid data for zooxanthellae (abbrev. to zoox in table) isolated from *T. gigas* (TG1, TG2 and TG3) and *T. squamosa* (TS). Animal TS was collected from OTI (values based on the single analysis of zooxanthellae from each animal).

Lab code	TG1 zoox	TG2 zoox	Average <i>gigas</i> zoox	<i>T.</i> TG3-albino zoox	TS zoox
% saturated	45.8	32.3	39.1	62.8	16.0
% unsaturated	54.2	67.7	60.9	33.6	82.7
%monounsaturated	8.3	6.2	7.2	6.8	6.8
%polyunsaturated	45.9	61.5	53.7	26.7	75.9
%unidentified	0	0	0	3.6	1.3
%identified	100	100	100	96.4	98.7
sat/unsat	0.8	0.5	0.7	1.9	0.2
Unsat index	200.4	260.3	230.4	115.7	353.9
16:0/16:1 ω 7	5.6	8.4	7.0	9.9	3.6
18:0/18:1 ω 9	0.9	0.6	0.8	3.1	0.2
18:0/18:1 ω 7	-	-	-	-	-
16:1 ω 9/16:1 ω 7	0	0	0	0	0
18:1 ω 9/18:1 ω 7	-	-	-	-	-
16:0/18:4	1.4	0.9	1.1	3.5	0.4
16:0/22:6 ω 3	6.5	4.2	5.3	18.7	0.7
% ω 3 acids	7.3	10.7	9.0	2.5	19.6
% ω 6 acids	7.5	12.2	9.9	5.8	7.3
%unknown	31.1	38.6	34.8	18.4	49.5
% ω 9	1.2	2.4	1.8	2.0	3.1
% ω 7	6.0	3.1	4.5	4.8	3.2
ω 6/ ω 3	1.0	1.1	1.1	2.3	0.4

Table 3.6. Fatty acid data for zooxanthellae isolated from *T. maxima*. Small animals refer to those with a shell size of less than 150 mm from umbo to shell margin and represent juvenile animals that have not reached sexual maturity. Abundance values represent the single analysis of zooxanthellae isolated from each animal.

Lab code	tm2zoox	tm3zoox	TM zoox	lab	tmlzoox	AVERAGE Small clams
% saturated	40.8	36.3	68.8		29.7	43.9
% unsaturated	59.1	63.7	28.3		70.3	55.4
% monounsaturated	13.0	6.0	9.6		7.9	9.1
% polyunsaturated	46.1	57.7	18.7		62.4	46.2
% unidentified	0	0	0		0	0
% identified	100	100	100		100	100
sat/unsat	0.7	0.6	2.4		0.4	0.8
Unsat index	212.7	259.6	88.1		285.2	211.4
16:0/16:1 ω 7	4.5	8.7	10.3		6.2	7.1
18:0/18:1 ω 9	0.6	0.6	1.7		0.6	0.9
18:0/18:1 ω 7	-	-	-		-	-
16:1 ω 9/16:1 ω 7	0	0	0		0	0
18:1 ω 9/18:1 ω 7	-	-	-		-	-
16:0/18:4	1.4	1.0	6.0		0.7	1.5
16:0/22:6	7.5	5.3	20.1		3.4	7.3
% ω 3 acids	6.0	6.6	2.7		8.4	5.9
% ω 6 acids	3.5	4.6	1.9		2.9	3.2
% unknown	36.6	46.5	14.1		51.1	37.1
% ω 9	6.2	3.1	4.3		2.0	3.9
% ω 7	6.8	2.9	5.3		3.5	4.6
ω 6/ ω 3	0.6	0.7	0.7		0.3	0.5

Table 3.7. Fatty acid data for zooxanthellae isolated from *T. maxima*. Large animals have a shell size of greater than 25 mm from umbo to shell margin and these animals are probably sexually mature. Abundance data represent the single analysis of zooxanthellae isolated from each animal.

Lab code	TM5a zoox	TM5b zoox	TM9B zoox	TM9A zoox	TM1b zoox	Average Large clams
% saturated	16.8	15.2	13.2	48.2	38.0	26.3
% unsaturated	82.6	84.8	85.8	51.8	61.6	73.3
monounsatur	6.7	5.0	3.4	17.4	14.8	9.4
polyunsatur	75.8	79.8	82.4	34.4	46.8	63.9
%unidentified	0	0	0	0	0	0
%identified	100	100	100	100	100	100
sat/unsat	0.2	0.2	0.1	0.9	0.6	0.4
Unsat index	344.3	360.2	374.3	172.4	217.9	293.8
16:0/16:1 ω 7	2.4	2.6	4.1	3.6	5.7	3.7
18:0/18:1 ω 9	0.2	0	0.3	0.7	0.4	0.4
18:0/18:1 ω 7	-	-	-	-	-	-
16:1 ω 9/16:1 ω 7	0	0	0	0	0	0
18:1 ω 9/18:1 ω 7	-	-	-	-	-	-
16:0/18:4	0.2	0.2	0.2	2.9	1.5	0.6
16:0/22:6	1.1	1.2	1.1	4.7	3.3	2.3
% ω 3 acids	10.5	9.1	9.9	9.7	10.7	10.0
% ω 6 acids	4.5	4.0	3.4	6.0	6.1	4.8
%unknown	60.9	66.7	69.1	18.8	30.1	49.1
% ω 9	3.2	1.8	1.4	7.2	10.3	4.8
% ω 7	3.5	3.2	2.0	10.2	4.5	4.7
ω 6/ ω 3	0.4	0.4	0.3	0.6	0.6	0.5

3.6.3 Fatty acids in zooxanthellae – an overview

The fatty acid profiles of zooxanthellae in all symbiotic species analysed were similar with 14:0, 16:0, 18:3 ω 6, 18:4, 18:5, 20:5 ω 3 and 22:6 ω 3 being major components. Acids 16:0 and 18:4 formed between 44 and 57% of the total acids. These findings are similar to previous studies of zooxanthellae, isolated from *T. maxima*, in which 16:0 and 18:4 formed 60% of the total acids and an unsaturation index of 290 and ω 6/ ω 3 of 1.1 (Bishop *et al.*, 1976). The fatty acid profiles of free living dinoflagellates are also similar to zooxanthellae from this

study with 16:0 and 18:4 forming between 24-46%, unsaturation indices of between 162 and 375 in free living dinoflagellates. The $\omega 6/\omega 3$ ratios in free living dinoflagellates are lower (≤ 0.2), than those found in zooxanthellae from this study, in five dinoflagellate species analysed (Ackman *et al.*, 1968; Reitan *et al.*, 1994; Harvey *et al.*, 1987; Hallegraeff *et al.*, 1991 and Zhukova and Aizdaicher, 1995) (Fig. 3.9). Both the analysis of zooxanthellae in this study, and the zooxanthellae in the study by Bishop *et al.* (1976), contrast markedly to the findings of Harland *et al.* (1991) and Kellogg and Patton (1983). Harland *et al.* (1991) and Kellogg and Patton (1983) isolated zooxanthellae from the anemones *A.viridis* and *C.gigantea*, that yielded mainly saturated acids (the UI were 54 and 14 respectively). There is an essential difference between the location of the zooxanthellae in the giant clams versus anemones, extracellular versus intracellular. The possibility that the zooxanthellae are held in slightly different nutritive states may explain the differences observed between the fatty acids isolated from Tridacnid clams and zooxanthellae isolated from anemones. Lipid concentrations in cultured algae typically increase as levels of nutrients fall, and the relative abundances of saturated acids also increase with a concomitant fall in PUFAs (Reitan *et al.* 1994). Furthermore it can not be ruled out that those samples previously analysed may also have suffered from PUFA loss by auto-oxidation and the results reported are sample preparation artefacts.

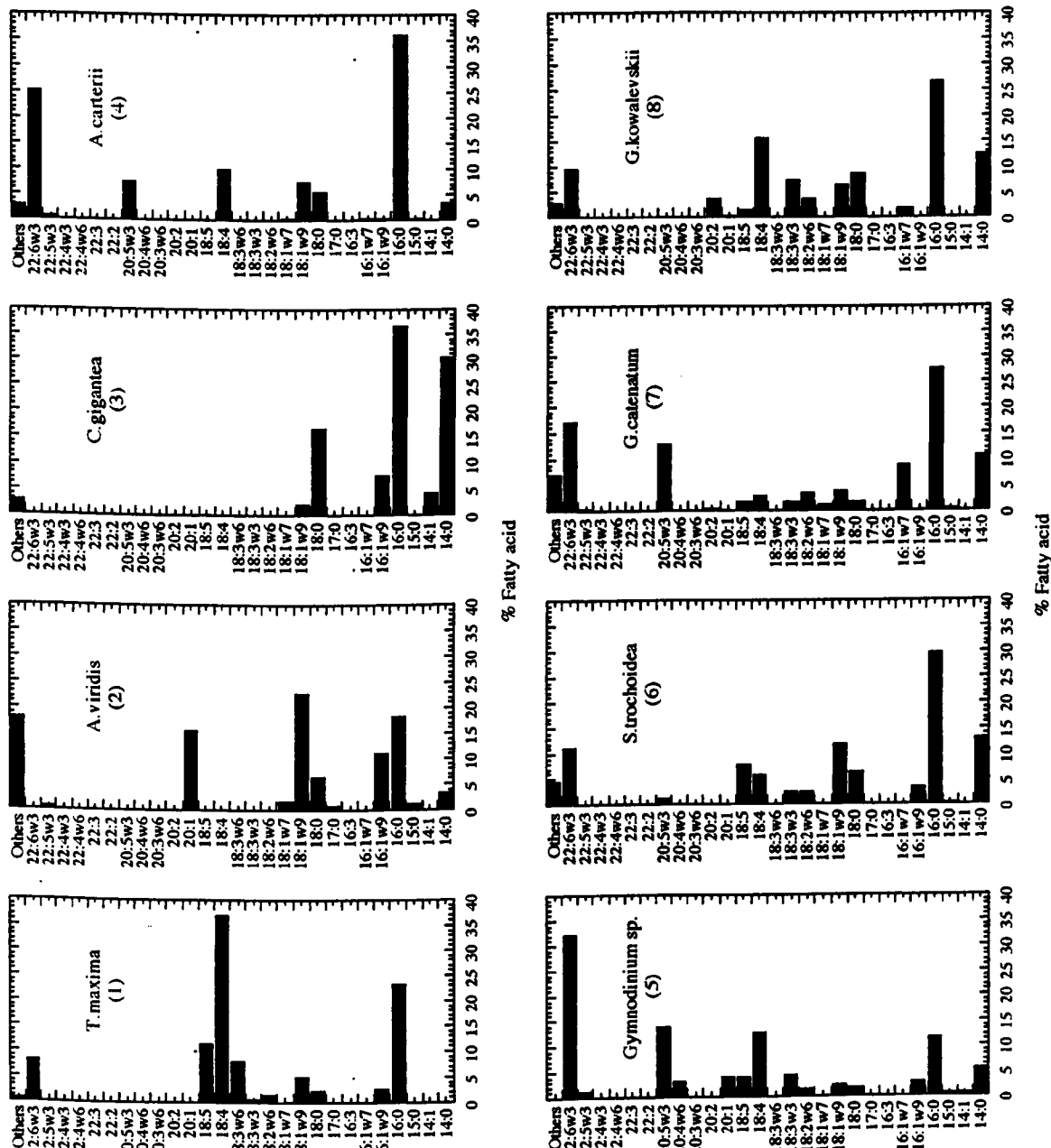


Fig. 3.9 Fatty acid profiles for zooxanthellae isolated from *T. maxima*, *A. viridis* and *C. gigantea* and the free living dinoflagellates *A. carterii*, *Gymnodinium* sp., *S. trochoidea*, *G. calenatum* and *G. kowalevskii*. Numbers under the species names in the plot refer to the source of the data. Key: 1. Bishop et al., 1976; 2. Harland et al., 1991; 3. Kellogg and Patton, 1983; 4. Ackman et al., 1988; 5. Reiten et al., 1994; 6. Harvey et al., 1987; 7. Hallegraeff et al., 1991; 8. Zhukova and Aizdalcher, 1995.

3.6.4 Bulk carbon isotopic composition of zooxanthellae

Bulk $\delta^{13}\text{C}_{\text{zooxanthellae}}$ values for isolated zooxanthellae were very similar from different animals of the same species and also from different species at $-15.9 \pm 1.0\text{‰}$ ($n=8$) and $-14.6 \pm 1.0\text{‰}$ ($n=10$) for *T. gigas* and *T. maxima* respectively (Figs. 3.6, 3.7 and 3.8). The zooxanthellae isolated from the albino *T. gigas* yielded a bulk $\delta^{13}\text{C}$ that was more ^{13}C -depleted, compared to its zooxanthellate contemporaries, at -17.5‰ . Only one zooxanthellae isolate from *T. squamosa* was available for analysis and had a bulk $\delta^{13}\text{C}$ of -16.0‰ . The total range for all zooxanthellae analysed, regardless of host species, was -12.4 to -17.5‰ ($n=20$). It is noteworthy that the average bulk $\delta^{13}\text{C}_{\text{zooxanthellae}}$ from small ($<100\text{mm}$) versus large ($\geq 200\text{mm}$) individuals of *T. maxima* is quite different, the large clam zooxanthellae being more ^{13}C -enriched than those from small animals (small= $-14.8 \pm 0.8\text{‰}$, large= $-12.9 \pm 0.5\text{‰}$). These values are significantly ^{13}C -enriched compared to previous reported isotopic data for zooxanthellae isolated from *T. maxima*, which yielded a $\delta^{13}\text{C}_{\text{zooxanthellae}}$ value of -23.3‰ (Black and Bender, 1976). However, the bulk $\delta^{13}\text{C}_{\text{zooxanthellae}}$ is consistent with that found in zooxanthellae isolated from corals (Land *et al.*, 1975; Risk *et al.*, 1994) Previous $\delta^{13}\text{C}_{\text{bulk}}$ values reported for the majority of marine algae range between -20 and -35‰ (Schidlowski *et al.*, 1986). There is a ^{13}C -depletion between aqueous HCO_3^- and CO_2 of between 9.2 and 6.8‰ in water temperatures of $0\text{--}30^\circ\text{C}$ (Deuser *et al.*, 1968). Assuming a typical warm water $\delta^{13}\text{C}$ value of -0.7‰ for CO_2 (O'Leary 1984) and a typical RuBisCo ^{13}C -discrimination found in C3 plants of -23‰ (Wong *et al.*, 1979; O'Leary, 1981; Raven, 1992), the bulk $\delta^{13}\text{C}_{\text{zooxanthellae}}$ values reported here, are more ^{13}C -enriched than might be expected, if the symbiotic algae are truly utilising the C3 photosynthesis pathway. Although zooxanthellae reside in tubules within the mantle tissue it is assumed that the clam haemolymph, which bathes the zooxanthellae, is in equilibrium with the ambient seawater. To date the technology does not exist to measure parameters, within the extremely small zooxanthellar tubules, to validate this assumption.

There are several reasons why zooxanthellae may exhibit $\delta^{13}\text{C}$ values that are not typical of C3 photosynthesis:

- (a) Zooxanthellae may not utilise the C3 pathway.
- (b) Zooxanthellae do utilise the C3 pathway in preference, but have to utilise bicarbonate at times of high irradiance when carbon dioxide supply cannot meet demand. Marine bicarbonate $\delta^{13}\text{C}$ values range between -1.0 to 2.0‰ whilst the typical $\delta^{13}\text{C}$ of dissolved carbon dioxide in warm waters is -0.7‰, this changes little with location. Use of bicarbonate would also result in more ^{13}C -enriched organic compounds.
- (c) The zooxanthellae are utilising another source of dissolved carbon dioxide besides that in ambient seawater. It is not currently possible to measure the $\delta^{13}\text{C}$ of dissolved carbon dioxide in the tubules. Several studies have suggested the use of host metabolic (respiratory) CO_2 by the zooxanthellae, which would have a different isotopic ratio to the CO_2 or HCO_3^- in the ambient water and is likely to have a similar isotopic composition to bulk whole body $\delta^{13}\text{C}$ of the host (Jones *et al.*, 1986)
- (d) The RuBisCo in zooxanthellae does not discriminate against ^{13}C to the same degree as RuBisCo from other C3 plants.
- (e) Zooxanthellae may be carbon limited. For instance the carbon dioxide supply may be insufficient. At such times the discrimination, usually exhibited by the enzyme, RuBisCo, cannot be fully expressed. As the pool of carbon dioxide becomes smaller the enzymatic discrimination for ^{13}C cannot be fully expressed, as it is necessary for the enzyme to utilise all available carbon substrate (^{13}C as well as the preferred ^{12}C) when the carbon pool is in short supply.

Although zooxanthellae have been reported to have functional enzymes for the C4 pathway, i.e. the enzymes phosphoenolpyruvate-carboxylase and pyruvate-carboxylase, the levels of these enzymes are low (Tyler and Trench, 1986). However their presence is not necessarily indicative of the operation of a C4 pathway since enzymes such as pyruvate-carboxylase are also involved in other metabolic functions such as lipogenesis (Scrutton and Young, 1972; Ting, 1976) It has been confirmed that 3-Phosphoglyceric acid is the first photosynthetic

product of zooxanthellae and that CO_2 is the active species preferred by zooxanthellar RuBisCo (Streamer *et al.* 1993; Yellowlees *et al.* 1993). Both of these observations support the view that the C3 pathway is the major route of carbon fixation in zooxanthellae.

Host respiratory CO_2 is a possible carbon source for zooxanthellae and may play an important role in the intracellular symbiotic algae of coral and other cnidarians. However, the role of respiratory CO_2 in the giant clam symbiosis (where the zooxanthellae are located extracellularly and therefore the CO_2 is not a direct source) is unknown. The $\delta^{13}\text{C}$ of respiratory CO_2 is typically $\sim 1\text{‰}$ depleted in ^{13}C compared to bulk tissue $\delta^{13}\text{C}$. The fixation of respiratory CO_2 , by RuBisCo however, if metabolic CO_2 is a significant input and the carbon pool is large, would result in bulk tissue $\delta^{13}\text{C}$ values more ^{13}C -depleted than those seen in this study. The significance of this is that if respiratory CO_2 is being used, it is likely to be only a small contributor to the total carbon pool.

The RuBisCo enzyme in higher plants and algae is composed of eight large and eight small protein subunits and is known as Form I RuBisCo (Knaff, 1989). In contrast Whitney *et al.* (1995) found that the RuBisCo in zooxanthellae resembled a Form II RuBisCo (a multimer of large subunits only), unique in eukaryotic photosynthetic algae, but present in some purple non-sulphur photosynthesising bacteria. The Form II enzyme, isolated from zooxanthellae, was found to be $\sim 65\%$ identical to that in *Rhodospirillum rubrum* and has also been found in free living dinoflagellates (Morse *et al.*, 1995; Rowan *et al.*, 1996). The significance of this finding is related to the isotopic discrimination associated with carboxylation in organisms utilising different RuBisCo forms. The reported isotope effect (k^{12}/k^{13}) associated with RuBisCo carboxylation for the Form I enzyme, measured by comparing the isotopic composition of the carbon 1 of the 3-phosphoglycerate to the source carbon dioxide (Fig.1.4 chapter 1), is 1.029 (Wong *et al.*, 1979; Roeske and O'Leary, 1985). This isotope effect has been shown to be much smaller for the Form II RuBisCo isolated from *R. rubrum* where a k^{12}/k^{13} of 1.0178 ± 8 has been reported (Roeske and O'Leary, 1985). Thus zooxanthellar RuBisCo may also exhibit a reduced ^{13}C -discrimination compared to the Form I enzyme. To date it has not been possible to do a similar experiment on zooxanthellar or dinoflagellate RuBisCo since

the enzyme deteriorates very quickly after cell lysis (Bugh and Sweeney, 1972; Whitney and Yellowlees, 1995). If the isotope effect is similar to that in *R. rubrum*, however, it cannot completely account for the $\delta^{13}\text{C}$ values observed in this study, unless the Form II RuBisCo discrimination is even smaller or other mechanisms exist to discriminate against ^{13}C .

One plausible reason for the $\delta^{13}\text{C}$ zooxanthellae signatures lies in the supply of inorganic carbon, either in the physical transport of the carbon species to the carboxylation site or the availability of a certain carbon species. O'Leary (1981) postulated that the small isotope fractionations seen in aquatic plants were probably due to the slow diffusion of CO_2 or slow permeability rates across membranes rather than operation of a C_4 fixation pathway. Disrupting the stagnant boundary layer by aeration increases the rate at which CO_2 reaches the carboxylation site so that aerated algal cultures also show ^{13}C -depleted bulk algal signatures compared to the $\delta^{13}\text{C}$ of non-aerated cultures (Johnston and Raven 1992).

The situation of zooxanthellae within the Tridacnid host means that haemolymph is the most direct source of inorganic carbon for carboxylation. It has been shown that clam haemolymph is in equilibrium with the ambient seawater in terms of its inorganic carbon pool (Yellowlees *et al.*, 1993). Although CO_2 is the preferred carbon species for RuBisCo, the levels of dissolved CO_2 in seawater and hence haemolymph are determined by the chemical equilibrium with carbonic acid shown in the following equation:



The reaction, $\text{H}_2\text{CO}_3 = \text{HCO}_3^{-} + \text{H}^{+}$, is an ionisation reaction which is kinetically rapid whereas the reaction $\text{H}_2\text{CO}_3 = \text{H}_2\text{O} + \text{CO}_2$ is kinetically slow. The hydration-dehydration reaction is very slow and the proportions of CO_2 , HCO_3^{-} and HCO_3^{-2} in solution at any one time are dependent on pH. At pH 8.2 to 8.3, typical in seawater, HCO_3^{-} is the dominant carbon species. This pH range equates to a total dissolved inorganic carbon concentration of about 2.2 mM when seawater is in equilibrium with atmospheric CO_2 . However the actual proportion of dissolved carbon dioxide is very small and may become rate limiting for photosynthesis in a closed system scenario which may occur in the zooxanthellar tubules. In this study the pH in clam haemolymph (syringe sampled from the haemal sinus) has been

found to vary between 7.7 and 8.2 and is dependent on the time of day, typically pH is higher at times of high irradiance, causing the CO₂ levels to fall (described in Chapter 4). DIC levels in the haemolymph of *T. gigas*, normally in the range of 1.8 mM, can be depleted in the haemolymph to as low as 0.6 mM (D.Yellowlees pers. comm.). Although it is unknown whether these pH measurements and indeed DIC levels are representative of those for the zooxanthellae within the tubules, this is the best estimate of conditions for the zooxanthellae that is presently available. As carbon dioxide has been found to be the preferred carbon species by zooxanthellar RuBisCo (Streamer *et al.*, 1993; Yellowlees *et al.*, 1993), at very low dissolved CO₂ levels, the zooxanthellae are potentially carbon limited. In such a scenario the isotopic discrimination normally associated with RuBisCo cannot be fully expressed which may explain the bulk zooxanthellar $\delta^{13}\text{C}$ isotopic signatures observed in this study.

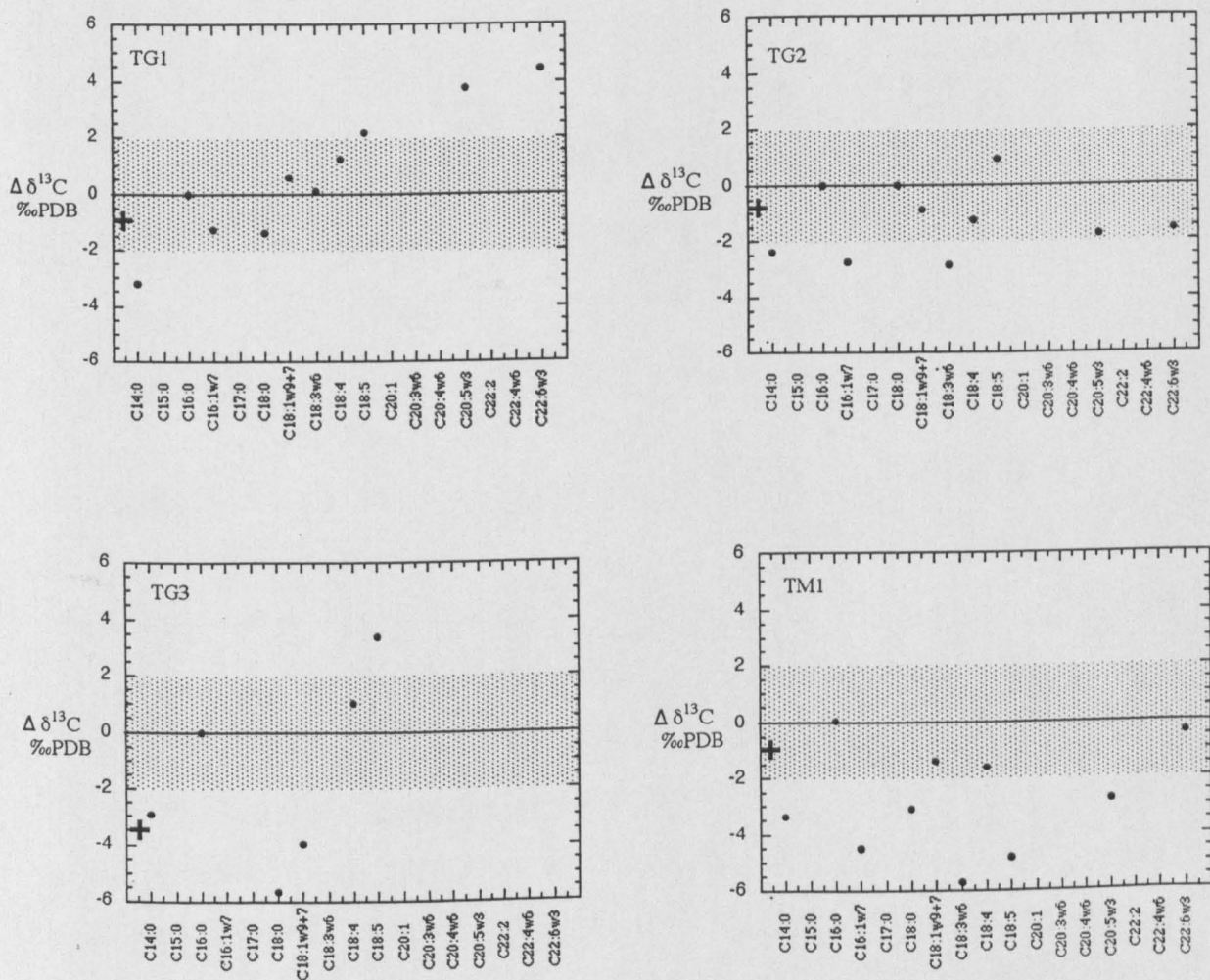


Fig. 3.10 $\delta^{13}\text{C}$ compositions of fatty acids versus that of the 16:0 acid in *T. gigas* (TG1,2 and 3) and *T. maxima* (TM1). The shaded area indicates a margin of ± 2 ‰ on the 16:0 $\delta^{13}\text{C}$ composition. Most elongation and desaturation transformations are thought to have small effects (Schouten *et al.*, 1998). Bulk $\delta^{13}\text{C}$ compositions of zooxanthellae relative to $\delta^{13}\text{C}16:0$ are plotted as crosses on the relevant plots.

3.6.5 CSIA of fatty acids from zooxanthellae

The $\delta^{13}\text{C}$ of the free fatty acids isolated from zooxanthellae range from -10.1 to -17.7‰ from *T. gigas*, with a slightly larger range for the albino animal (-10.6 to -19.7‰) and -13.8 to -19.1‰ from *T. maxima*. An interesting feature of these data is that several fatty acids are only minimally ^{13}C -depleted or actually ^{13}C -enriched, compared to bulk $\delta^{13}\text{C}_{\text{zooxanthellar}}$ values. All four symbiotic animals analysed for bulk zooxanthellar $\delta^{13}\text{C}$ yielded 16:0 fatty acids that were ^{13}C -enriched compared to bulk $\delta^{13}\text{C}_{\text{zooxanthellar}}$ values (Fig. 3.10). In the animal TG1, for instance, the isotopic composition of the 18:5 fatty acid is -12.4 ± 1.4 , some 2.9‰ ^{13}C -enriched compared to a bulk $\delta^{13}\text{C}_{\text{zooxanthellar}}$ value of -15.3 ± 0.2 , which is greater than would be expected from the experimental errors on the isotopic analyses.

The biosynthesis of fatty acids in all eukaryotic systems involves three main processes: the biosynthesis of palmitate from acetyl-CoA, chain elongation and desaturation. Lipids typically have $\delta^{13}\text{C}$ values that are ^{13}C -depleted compared to bulk tissue by 4 to 12‰ (Park and Epstein, 1961; Abelson and Hoering, 1961; Parker, 1962, 1963 and 1964; Degens, 1969). This difference lies in the kinetic isotopic effect exerted by the enzyme pyruvate dehydrogenase (actually a complex of enzymes) which is responsible for the synthesis of acetyl-CoA from pyruvate during lipid synthesis (DeNiro and Epstein, 1977; Melzer and Schmidt, 1987; Monson and Hayes, 1980). Pyruvate is primarily supplied either from glycolysis in animals, or the Calvin cycle during photosynthesis in plants. In animals pyruvate may also be produced from the degradation of alanine, when it accumulates in excess of requirements, by the process of transamination. There is apparently no significant isotope effect in the conversion of glucose to pyruvate (Abelson and Hoering, 1961). Although the isotope effect of the pyruvate dehydrogenase complex *in vivo* was not initially measured, DeNiro and Epstein (1977) measured the effect of the closely related enzyme pyruvate decarboxylase isolated from yeast. The enzymatic reaction yielded a 7‰ depletion in ^{13}C for the acetyl-CoA compared to the

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zooxanthellae. Although it is believed that algal mitochondrial acetate is a more likely source for fatty acid biosynthesis the host source cannot be ruled out. It is possible that the degradation of phytol (as chlorophylls) to form acetate may be a preferred source of acetate for the synthesis of fatty acids by algae (Ackman *et al.*, 1971). All fatty acid synthesis is carried out in the plastids in plants and algae, where the pyruvate dehydrogenase complex is sited, thus mitochondrial acetate is not a direct source for fatty acid biosynthesis. This is made more problematic by the fact that acetyl-CoA produced in the mitochondrion cannot cross the mitochondrial membrane in that form, and can only do so by conversion to citrate. Citrate is then reconverted to acetyl-CoA in the cytosol for subsequent use. However, the isotopic fractionation associated with the citrate shuttling of the acetyl-CoA has not been studied.

Using the pyruvate \Rightarrow acetyl-CoA scheme, it is more difficult to explain how fatty acids can become isotopically ^{13}C enriched compared to bulk tissue $\delta^{13}\text{C}$. The use of the acetate \Rightarrow acetyl-CoA scheme, however, could explain ^{13}C -enriched acids, especially considering the possible source of host derived acetate, as fatty acids produced by this route have smaller ^{13}C -depletions ($\sim 1\%$) from the source substrate than directly from pyruvate ($\sim 7\%$) (DeNiro and Epstein, 1977; Melzer and Schmidt, 1987), and in addition the host acetate may be isotopically ^{13}C -enriched compared to zooxanthellar acetate. The reciprocal exchange of acetate and fatty acids in symbiotic associations, has been suggested before, by Patton *et al.* (1977). Indeed Blanquet *et al.* (1979) showed that under light conditions up to 70% of ^{14}C -labelled acetate was incorporated into the 16:0 and 18:1 acids appearing in the host. The ^{13}C -enriched acids in this study would therefore appear to support this idea. There is however an alternative hypothesis that the ^{13}C -enriched fatty acids that are present in zooxanthellar isolates are actually fatty acids which have been translocated to the algal partner in the association and not vice versa. Intriguingly this may explain why long chain fatty acids are not consistently ^{13}C -depleted as expected from simple elongation of the fatty acid 16:0.

PUFAs which are ^{13}C -enriched have recently been reported in cultured algae, in which C20 and C22 PUFAs were found to be up to 8‰ ^{13}C -enriched compared to that of the C16 fatty acids (Schouten *et al.*, 1998). Of the fourteen algae cultured, only two species showed such ^{13}C -enrichments, both of which were dinoflagellates of the species *Gymnodinium* and *Amphidinium*. Although Schouten *et al.* (1998) offered no explanation as to why this may be so, it does suggest a biosynthesis difference in dinoflagellates compared with other algae, that has nothing to do with the use of host derived acetate since these dinoflagellates are free living and not within symbioses. The mechanism for ^{13}C -enrichment of fatty acids in zooxanthellae is apparently very complex. This issue is discussed further when the carbon isotopic compositions of fatty acids isolated from non-symbiotic bivalves are considered (section 3.10.6).

3.6.6 Lipids in cultured zooxanthellae

Culture Experiments

Zooxanthellae were isolated from five *T. gigas* (Clams 1 to 5) collected from Pioneer Bay, Orpheus Is. Each zooxanthellae isolate was then split into three aliquots. One of the aliquots was frozen immediately (herein referred to as 'freshly isolated zooxanthellae'). The two remaining isolates were divided into two 250ml conical flasks containing 100ml f2 culture medium, antibiotics being omitted (f2 medium preparation in Appendix). One set of flasks had their neck openings covered with foil (herein referred to as 'cult zoox not aerated') whilst the second set was aerated (herein referred to as 'cult zoox aerated') using a small aquarium pump and clean silicone tubing immersed directly into the culture medium. Both sets of flasks were incubated under two light sources, a blue light source and GroLux daytime light. A day/night cycle was used for the 10-day incubation with a 14hour-daylight period, the two light sources provided circa 200 μS irradiance. All equipment and culture medium were autoclaved before use.

The cultures were checked microscopically after the incubation period and were found to have negligible bacterial cells in all cultures. All cultures were transferred to centrifuge vessels and centrifuged at 2500rpm for 5 min at room temperature. The cultures were washed in filtered sea water after removal of the culture medium, before freezing at -20°C. Zooxanthellae isolated from a clam maintained in filtered sea water for 7 days (herein referred to as 'starved clam zoox') and zooxanthellae from two isolated clams maintained in the dark for 7 days (herein referred to as 'dark clam zoox') were also collected for comparative analysis.

Total lipid

The total lipid content for freshly isolated zooxanthellae from *T. gigas* was $7.4 \pm 1.1\%$ wet weight (n4). Cultured zooxanthellae exhibit much lower lipid contents at $2.6 \pm 0.8\%$ (n5) and 2.9 ± 1.1 (n5) for aerated and non-aerated cultures respectively (Table 3.8).

Table 3.8. Lipid contents and bulk $\delta^{13}\text{C}$ data for isolated zooxanthellae versus cultured zooxanthellae, either cultured with aeration from a small aquarium pump or not aerated.

Sample	%lipid of wet wt	Bulk $\delta^{13}\text{C}$ (‰)
Cult zoox aerated (Clam 1)	1.3	-19.0
Cult zoox aerated (Clam 2)	2.6	-15.4
Cult zoox aerated (Clam 3)	2.4	-16.6
Cult zoox aerated (Clam 4)	3.1	-18.9
Cult zoox aerated (Clam 5)	3.5	-18.1
Average	2.6 ± 0.8	-17.6 ± 1.6
Cult zoox not aerated (Clam 1)	4.7	-16.5
Cult zoox not aerated (Clam 2)	2.5	-15.0
Cult zoox not aerated (Clam 3)	2.5	-16.5
Cult zoox not aerated (Clam 4)	3.1	-17.9
Cult zoox not aerated (Clam 5)	1.8	-16.9
Average	2.9 ± 1.1	-16.5 ± 1.0
Freshly isolated zoox (Clam 1)	6.1	-16.8
Freshly isolated zoox (Clam 2)	-	-17.8
Freshly isolated zoox (Clam 3)	7.1	-16.1
Freshly isolated zoox (Clam 4)	8.7	-17.1
Freshly isolated zoox (Clam 5)	7.7	-16.9
Average	7.4 ± 1.1	-16.9 ± 0.6
Dark clam zoox (1)	9.5	-15.1
Dark clam zoox (2)	7.2	-17.7
Average		-16.4 (n=2)
Starved clam zoox	8.4	-18.0 (n=1)

Table 3.9 Fatty acid data for fresh isolated and cultured zooxanthellae from *T.gigas*. Data represent average value for each category: 5 animals for freshly isolated zooxanthellae and the same 5 animals for each of the cultured zooxanthellae, one animal for the starved clam and two for the dark clam zooxanthellae.

	<i>Cultured Aerated</i>	<i>Isolated Zooxanthellae</i>	<i>Cultured Non-aerated</i>	<i>Starved clam Zooxanthellae</i>	<i>Dark clam Zooxanthellae</i>
% saturated	32.1	21.5	39.4	24.3	21.5
% unsaturated	67.9	78.5	60.6	75.7	78.5
%monounsaturated	10.5	6.5	8.5	9.1	7.8
%polyunsaturated	57.5	71.9	52.1	66.6	70.8
%unidentified	0	0	0	0	0
%identified	100	100	100	100	100
sat/unsat	0.5	0.3	0.7	0.31	0.3
Unsat index	278	321	244	308	324
16:0/16:1 ω 7	5.8	4.5	9.2	4.6	4.3
18:0/18:1 ω 9	0.5	0.7	0.9	0.4	0.6
18:0/18:1 ω 7	-	-	-	-	-
16:1 ω 9/16:1 ω 7	0	0	0	0	0
18:1 ω 9/18:1 ω 7	-	-	-	-	-
16:0/18:4	1.1	0.6	1.3	0.7	0.6
16:0/22:6	1.9	2.2	3.4	2.2	2.0
% ω 3 acids	17.4	13.0	12.0	17.1	16.4
% ω 6 acids	3.1	7.7	3.1	4.6	5.2
%unknown	37.0	51.2	37.0	44.9	50.0
% ω 9	5.5	2.0	4.7	4.1	2.3
% ω 7	5.0	4.5	3.8	4.9	4.6
ω 6/ ω 3	0.2	0.6	0.3	0.3	0.3

Fatty acids isolated from cultured vs. isolated zooxanthellae

Freshly isolated zooxanthellae yielded similar fatty acid profiles (to those of TG1 and TG2 described previously). The fatty acids 18:4, 16:0 and 18:5 dominate the fatty acid profiles, Fig.3.11 (page 130). There were no significant difference in the fatty acid profiles of freshly isolated zooxanthellae compared with those zooxanthellae isolated from the starved clam or zooxanthellae isolated from the clams kept in the dark. Unsaturation indices, 16:0/18:4 and 16:0/22:6 ω 3 ratios from zooxanthellae isolated from the dark and starved clams are within experimental error of the freshly isolated zooxanthellae. However, ω 6/ ω 3 ratios are somewhat lower for the zooxanthellae from the starved and dark clams clam (ω 6/ ω 3 ratios = 0.3) compared to 0.6 of the freshly isolated zooxanthellae (Table 3.9).

Fatty acid results for the starved clam and dark clam zooxanthellae might indicate that the zooxanthellae continue to synthesis the same fatty acids whether the clam is in the light, filter feeding or in the dark, so the biosynthesis is independent of any clam input and also that the algae are not necessarily dependent on light for fatty acid synthesis. However it should be noted that the incubation times for the two experiments (starved and dark clams) were, of necessity short, to prevent undue stress to the animal, and may have been of insufficient time for the algae to exhibit marked changes in the fatty acid synthesis.

Zooxanthellae, cultured for 10 days, show subtle changes in their fatty acid content compared to the freshly isolated zooxanthellae samples (Fig. 3.11). Although the combined content of the acids 16:0 and 18:4 remains high in isolated and cultured zooxanthellae, comprising >56%, the relative proportion of 16:0 to the total is higher in the cultured zooxanthellae. There is clearly a higher total saturated acid content in the cultured compared to freshly isolated zooxanthellae (Table 3.9). The UI decreases with aeration as do the 16:0/16:1 ω 7, 18:0/18:1 ω 9, 16:0/18:4, 16:0/18:5 and 16:0/22:6 ω 3 ratios in the cultures. Although dissolved carbon dioxide contents were not measured during the experiment certain assumptions can be made about the DIC in the experiments based on other empirical data. Johnston and Raven

(1992) found that autoclaved culture medium typically has a very low DIC content which re-equilibrates slowly to around 1.1 mM after two days, with well aerated medium reaching a level slightly higher than the air-equilibrium value of 1.9 mM. Presumably the re-equilibration with atmospheric CO₂ is slower than the CO₂ uptake by the zooxanthellae and although the non-aerated culture medium will have commenced the experiment at around 1.0 mM, the DIC will have reduced markedly as the zooxanthellae continue to photosynthesise. However, the aerated culture will have retained a much higher DIC level and the changes that are apparent in the fatty acid synthesis could be due to changes in either available DIC or O₂ levels. *T. gigas* haemolymph (clam blood) DIC levels averaged 1.6 ± 0.4 mM (n=48), (six animals were sampled each eight times during a twenty-four cycle, chapter 4) during the course of an experiment for this study. Assuming a similar level of DIC (1.6mM) for the freshly isolated zooxanthellae there does not appear to be a systematic change in UI or other parameters with DIC content.

Carbon isotope composition of cultured vs. isolated zooxanthellae.

The average bulk $\delta^{13}\text{C}$ results were $-16.9 \pm 0.6\text{‰}$, $-16.5 \pm 1.0\text{‰}$ and $-17.6 \pm 1.6\text{‰}$ for freshly isolated zooxanthellae, cultured zooxanthellae (not aerated) and cultured zooxanthellae (aerated) respectively. Analysis of the five pairs of cultured samples demonstrated a general trend of reducing $\delta^{13}\text{C}$ with increasing aeration (from predicted DIC) in four of the sets, one set remained the same Fig. 3.12, (page 131).

This observation agrees with a hypothesis that the isotopic composition of zooxanthellae would change with the level of available DIC. This data set therefore supports earlier evidence, that the zooxanthellae in *T. gigas* are apparently carbon limited, and that the discrimination of zooxanthellar RuBisCo is not fully exhibited in *in vivo* conditions. Although it is believed that carbon is less likely to be limiting for algae than nitrogen and phosphate other experiments have found that increasing the DIC to cultured algae can increase their growth rates (Raven and Johnston, 1993). Carbon limitation may be a function of the slow

diffusion of CO₂ in water for free living or cultured algae and aeration may disturb the boundary layer of an organism thus ensuring fresh supplies of DIC. The supply of DIC in the haemolymph of zooxanthellae is different to free living or cultured algae, because of the symbiotic arrangement, and it is unknown whether the actual DIC available to the zooxanthellae within the tubules is truly in equilibrium with ambient seawater, as measurements have only been carried out on clam haemolymph (this study and Rees et al., 1993). These culture experiments do not, however, address the question of whether the algae possess RuBisCo, with a similar discrimination to *R. rubrum*. The ¹³C-discrimination of the Form II RuBisCo in zooxanthellae may account for the ¹³C-enriched values reported (section 3.6.4), however, the RuBisCo isolated so far, has proved to be short-lived so that isotopic fractionation experiments have yet to be carried out.

CSIA of fatty acids from cultured zooxanthellae

The δ¹³C compositions of the 16:0 acid extracted from freshly isolated and cultured zooxanthellae are given in Table 3.10. The δ¹³C values for freshly isolated zooxanthellae of five clams taken from the reef are all very similar and range from -19.0 to -20.9‰, those from the cultured zooxanthellae are a little more variable: cultured but not aerated -18.8 to -22.4‰, cultured and aerated -19.0 to -24.5‰. The difference in isotopic composition of the 16:0 fatty acid from the freshly isolated zooxanthellae versus the cultured zooxanthellae are -0.6 to +5.2‰ for the aerated culture and -0.6 to +3.4‰ for the non-aerated culture. The isotopic composition difference between aerated and non-aerated cultures for the 16:0 fatty acid is -3.3 to +0.2‰. Unfortunately there is insufficient data for statistical analysis. The isotopic composition change (for 16:0) for aerated culture versus non-aerated culture is not uniform for all the clam zooxanthellae isolated and may well indicate that the aeration was not uniform for each culture flask.

Table 3.10. $\delta^{13}\text{C}$ compositions of the 16:0 fatty acid in freshly isolated versus cultured (aerated and non aerated) zooxanthellae. One culture was lightly covered only whilst the second was aerated from a small aquarium pump. The starved animal was kept in FSW in the light for 7 days prior to sampling. The dark animals were maintained in a lightproof container fed with flowing sand-filtered seawater. All values are ‰PDB, errors $\pm 1\sigma$ and nd represents no data.

	Clam zoox $\delta^{13}\text{C}$ ‰ PDB	No aeration $\delta^{13}\text{C}$ ‰ PDB	Aeration $\delta^{13}\text{C}$ ‰ PDB
1	-20.9 ± 0.6	-21.2 ± 1.0	-24.5 ± 0.5
2	nd	-18.8 ± 0.6	-20.1 ± 0.5
3	-21.0 ± 0.7	nd	-20.4 ± 0.4
4	-19.0 ± 1.3	-22.4 ± 1.1	-24.2 ± 0.7
5	-19.8 ± 0.7	-19.2 ± 0.5	-19.0 ± 0.4
Starved	-25.9 ± 1.4		
Dark 1	-16.6 ± 0.8		
Dark 2	-23.0 ± 1.0		

Isotopic signatures for the 16:0 fatty acid of zooxanthellae from the two dark clams were inconsistent at -16.6 ± 0.8 and -23.0 ± 1.0 ‰, a difference of 6‰. The bulk $\delta^{13}\text{C}$ values showing a similar trend (Table 3.8), clam 2 exhibiting a 3‰ more ^{13}C -depleted value in the bulk $\delta^{13}\text{C}$ zooxanthellar composition than clam 1. The discrepancy between the isotopic signatures of the two dark clam's zooxanthellae may be explained by the relative positions of the animals in the experimental tank. The difference may be a function of the CO_2 availability to the animal/zooxanthellae furthest away from the water inlet (clam 1), which may have suffered some DIC depletion in its seawater supply because of DIC utilisation by clam 2 zooxanthellae. This aspect may have been exacerbated by the release of waste products, such as ammonia into the water by the clam upstream, thus increasing the seawater pH and concomitantly decreasing the CO_2 content.

An interesting observation is the ^{13}C -depleted value for the zooxanthellar 16:0 fatty acid isolated from the starved clam and merits further investigation. Although the seawater was filtered (0.45microns), it was originally sourced from Pioneer Bay, Orpheus Island. The experimental tank was also housed out of doors and consequently would not be affected by

local $\delta^{13}\text{C}$ changes due to respiratory CO_2 from lab workers etc. Thus, one might expect that the tank seawater would have a similar $\delta^{13}\text{C}_{\text{DIC}}$ value to that in seawater in Pioneer Bay. However, zooxanthellae from the starved animal, which sampled seawater without the POM component, has a dissimilar $\delta^{13}\text{C}_{16:0}$ value to the $\delta^{13}\text{C}_{16:0}$ value in zooxanthellae isolated from animals directly from the reef flats. The bulk $\delta^{13}\text{C}_{\text{zooxanthellae}}$ was very similar in zooxanthellae isolated from the starved animal compared with zooxanthellae isolated from the animals taken directly from the reef. However, $\delta^{13}\text{C}_{16:0}$ of zooxanthellae isolated from the starved animal has yielded a more ^{13}C -depleted $\delta^{13}\text{C}_{16:0}$ value than the 16:0 fatty acid isolated from clams directly from the reef. Presumably because the clam is starved there are no catabolic products from filter feeding, such as acetate, for zooxanthellae to utilise for fatty acid synthesis, if indeed they do. This is circumstantial evidence for the use of host catabolic acetate by the zooxanthellae and further experimentation is warranted to check this.

3.7 LIPIDS IN SYMBIOTIC BIVALVES

3.7.1 Sample details

The lipid results for tissues from five symbiotic species are presented in this section: whole-body and individual tissues from *T. maxima*; individual tissues from *T. gigas* (n=3), including a partial albino specimen; individual tissues from one *T. squamosa* specimen; and the mantle tissue from one specimen of *H. hippopus*. The numbers of animals used for each analysis are given in the relevant sections. Individual tissue samples typically were mantle, muscle and digestive mass or combined remaining tissues, although additional tissues were taken for the species *T. gigas*. (Sample details and numbers are provided in the appendices).

3.7.2 Lipid compositions in symbiotic bivalves

Total lipids from symbiotic bivalves

Average whole-body lipid composition for twelve individuals of the symbiotic species *T. maxima* from patch reefs in One Tree lagoon was $8.2 \pm 3.5\%$ (reproducibility of total lipid concentrations was typically 3% or better from the triplicate analysis of each of the 12 individuals). Total lipid compositions for adductor, mantle, gills, digestive mass, combined remaining tissues were 0.5-5.9%, 5.8-7.5%, 4.3-23.2%, 7.9-8.1%, 7.1-19.1% respectively in the symbiotic bivalves (Fig. 3.13).

The *T. gigas* animal, which had been held in the JCU aquarium, shows differences in its tissue lipid content compared to its contemporary from Orpheus. Although the level of lipid in the adductor and mantle is similar, the lipids are higher in the digestive mass in the aquarium animal, apparently an indication of the different dietary lipid contents available to each animal. A noteworthy point is that the lipid concentrations in the gills and zooxanthellae in the animal from the JCU tank are 50% of the contents seen in the animal immediately sampled on collection. This confirms that environmental factors have a great influence on the lipid levels in these animals and their zooxanthellae. For example, irradiance levels are dissimilar in the two cases and water quality is also likely to be different, nutrient and POM levels are both higher for the aquarium animal. The build up of ammonia in the aquarium is also a distinct possibility. The two *T. gigas* animals taken directly from Orpheus were from maricultured stock and from the same cohort, the only obvious difference between the animals was that of the partially bleached mantle. Although digestive mass and mantle lipid content was similar in these two animals the albino clam had almost 4 times the lipid in its gills compared to the normal clam. Adductor lipids were also twice as high in the albino clam as in the normal animal. As digestive mass lipid content is almost identical it suggests that these animals were probably sourcing a similar diet as regards to lipid composition. The presence of lipid-rich zooxanthellae in the *T. gigas* does not affect the overall lipid content of the mantle containing

them, thus confirming an earlier assumption that the zooxanthellae are present at a concentration of <10% of the total mantle.

Free fatty acids isolated from T. maxima

Saturated fatty acids formed between 43.2-72.1% of the whole-body tissues in twelve *T. maxima* collected from twelve locations in OT lagoon (Fig. 3.14). Generally the pattern of fatty acid distribution in *T. maxima* whole tissues was fairly similar for the fatty acids 18:0 and longer carbon chains, however a marked variation was seen in the ratio of 16:0/16:1 ω 7 (1.5-9.5%) and in the abundance of 14:0 for the twelve animals. The whole-body fatty acid profiles and $\delta^{13}\text{C}$ values given in Fig. 3.14 have been used to infer nutritional sources for *T. maxima* in section 3.8. Individual tissue saturated fatty acids from *T. maxima* comprised 28.6-53.8% and 30.4-45% in mantle tissue, 29.1-59.2% and 36.8-38.5% in muscle from small and large clams respectively. Combined remaining tissues in *T. maxima* had the largest range at 37.2-70.85% lipid for all clams. Unsaturation indices ranged from 59-272 for all tissues in *T. maxima*. ω 6/ ω 3 ratios were between 0.3 and 1.5% for all tissues from *T. maxima* except for CRT which was much higher at 3-8.9% (Fig. 3.15, Data tables in appendices).

Free fatty acids isolated from T. gigas

Saturated fatty acids from the two fully zooxanthellate *T. gigas* (TG1 and TG2) formed 50% and 46.2% of the total fatty acids in mantle, 39.4% and 53.4% in adductor muscle, 53.6% and 56% in gills, 62.2% and 53.4% in digestive mass tissues. The partial albino *T. gigas* generally had slightly lower tissue saturated fatty acid contents than its zooxanthellate contemporaries and yielded saturated fatty acid contents of 34.5% in bleached mantle, with 39% and 45.4% in the normal and lateral mantle respectively. The ω 6/ ω 3 ratios were between 1.0 and 4.0 for all individual tissues from the fully zooxanthellate *T. gigas* (TG1 and TG2), however the range for the albino *T. gigas* was smaller at 1.0-2.0 (Figs. 3.16, 3.17 and 3.18).

Free fatty acids isolated from T. squamosa and H. hippopus

Mantle tissues from *T. squamosa* and *H. hippopus* species contained 29.9% and 34.7% saturated fatty acids respectively, muscle from *T. squamosa* had a higher content of 54.3%. Unsaturation indices from tissues from both animals were between 124-233 and $\omega 6/\omega 3$ ratios 0.5-1.1 (Figs. 3.19 and 3.20).

3.7.3 Fatty acids in symbiotic bivalves – an overview

Saturated fatty acids

The major saturated fatty acid present in all of the symbiotic bivalves, *T. gigas*, *T. maxima*, *T. squamosa* and *H. hippopus*, was palmitic acid (16:0). This fatty acid was also the major fatty acid in all clam tissues. Palmitic acid formed 31.7-56.9% of total fatty acids in whole-body analyses from 12 individuals of the species *T. maxima* (collected from One Tree Lagoon) and 20.0-49.7% in adductor muscle, 18.4-45.7% in mantle tissue and 23.1-60.5% in combined remaining tissues of this species. *T. squamosa* also possessed 16:0 as its major fatty acid in all tissues (mantle 21.0%, adductor 43.3%, gills 23.9% and CRT 32.8%). Isolated mantle tissues of *T. gigas* yielded between 23-41% palmitate, with the lowest concentration in bleached mantle from the partial albino. Other *T. gigas* tissues had between 26-56% in adductor muscle, 24-43% in gills and 22-46% in digestive mass of this fatty acid. Other saturated fatty acids, 14:0, 15:0, 17:0 and 18:0, typically formed between 1-10%, of the total fatty acids in *T. gigas*, *T. maxima*, *T. squamosa* and *H. hippopus* tissues, with 14:0 and 18:0 predominating. No saturated fatty acids above 18 carbons were found in the various tissues from symbiotic bivalves (Figs. 3.14 to 3.20).

Monounsaturated acids

Six monounsaturated fatty acids were identified in various tissues from the symbiotic animals; 14:1, 16:1 ω 7, 16:1 ω 9, 18:1 ω 7 and 18:1 ω 9. The fatty acid 14:1 occurred rarely, the only

mantle tissue to contain this fatty acid (0.3%) was from *H. hippopus*, generally this fatty acid comprised less than 2.5% when present in individual clam tissues. As in the non-symbiotic species studied, 16:1 ω 9 was found rarely and the 16:1 ω 7 isomer was always the dominant isomer. Mantle tissues from the species *T. maxima*, *T. gigas*, *T. squamosa* and *H. hippopus* possessed 16:1 ω 7 in the range 4.1-8.3%. In the species *T. maxima*, *T. gigas*, *T. squamosa* 16:1 ω 7 was present in the range 2.4-14.6%, 3.0-25.2% and 2.9-4.5% in adductor muscle, combined remaining tissues/digestive mass and gill tissue respectively. The *T. gigas* albino clam fatty acid profile for 16:1 ω 7 was not significantly different from the normal individuals. 18:1 ω 9 was always in greater concentration than the 18:1 ω 7 isomer in all tissues from the symbiotic species (Figs. 3.14 to 3.20).

Polyunsaturated acids

The major polyunsaturated fatty acids (PUFAs) present in various tissues of symbiotic animals were 18:3 ω 6, 18:4, 18:5, 20:4 ω 6, 20:5 ω 3, and 22:6 ω 3. Whole-body tissues contained 20:4 ω 6 and 22:6 ω 3 as the most predominant ($\leq 10\%$) PUFAs. Varying concentrations of 18:3 ω 6, 18:4, 18:5, 20:2, 20:3 ω 6, 20:5 ω 3, 22:2, 22:4 ω 6 and 22:5 ω 3 were also present with each of these fatty acids typically composing less than 5% of the total. In *T. gigas* relative concentrations of 18:3 ω 6 and 18:4 were higher in mantle than other tissues from these animals, whereas concentrations of 20:3 ω 6, 20:4 ω 6 and 20:5 ω 3 were lower in mantle tissue than other tissues. The data for the albino *T. gigas* are similar, however the bleached and normal mantles have higher relative amounts of the 20:x acids than for the lateral mantle sample (Figs. 3.14 to 3.20).

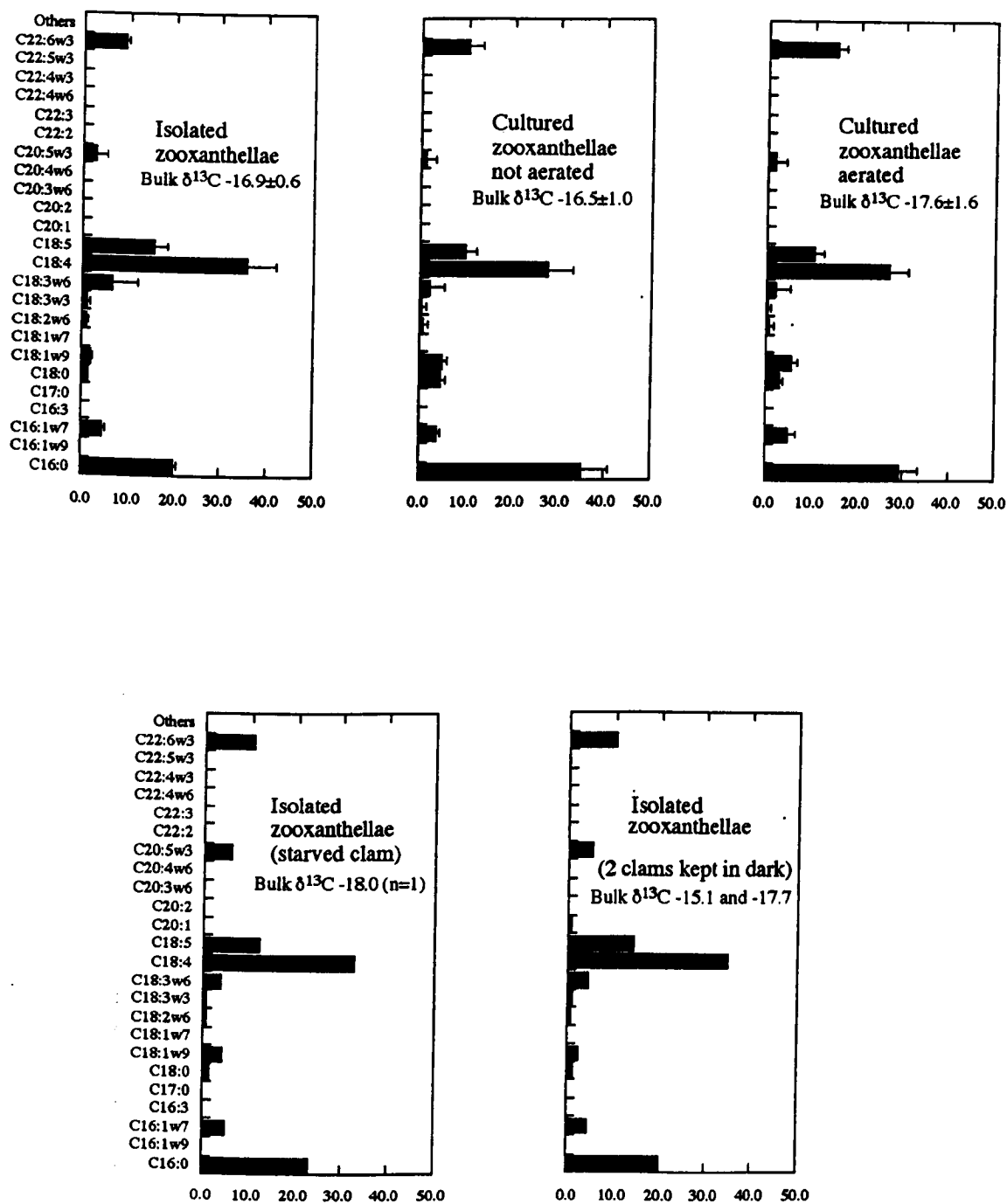


Fig. 3.11 Fatty acid profiles for isolated zooxanthellae and cultured zooxanthellae from *T. gigas*. The "starved" clam was maintained in FSW for 7 days. The "dark" clam was maintained in a black plastic bin fed with sand filtered seawater for 7 days. Bulk $\delta^{13}\text{C}$ represent average values for zooxanthellae isolated from five animals unless otherwise indicated in parenthesis. Isotopic data expressed as $\delta^{13}\text{C}$ ‰.

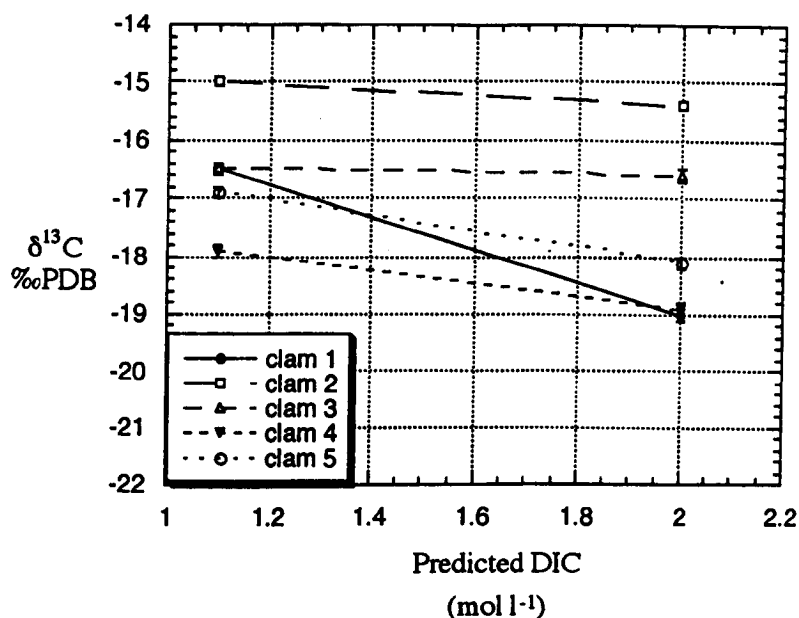


Fig. 3.12 Bulk $\delta^{13}\text{C}$ of cultured zooxanthellae at two different DIC concentrations, level of DIC increased by aeration, source carbon dioxide common to both.

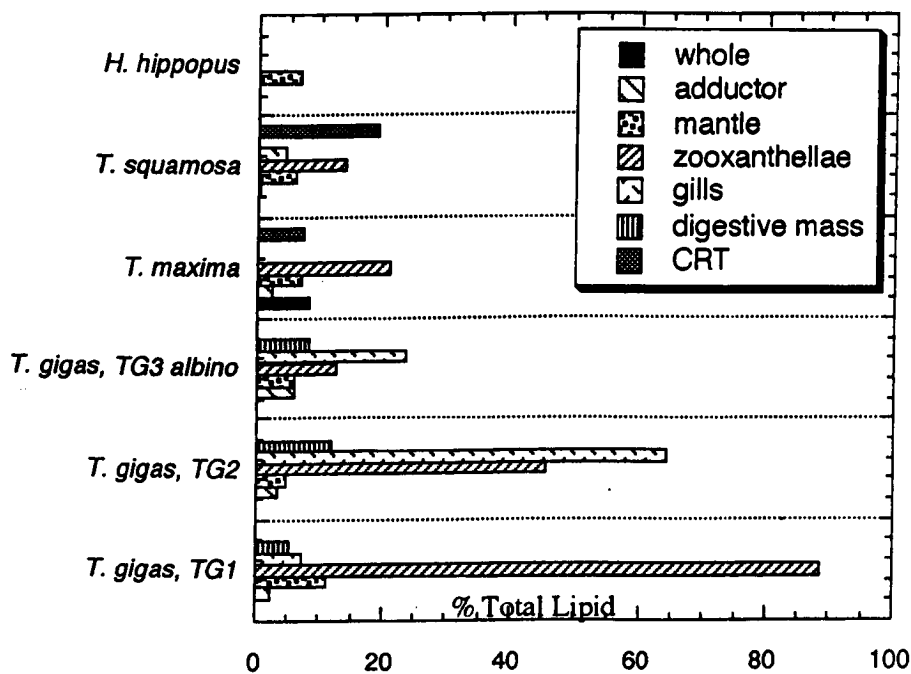


Fig. 3.13 Lipid compositions of tissues from symbiotic bivalves, collected from OT lagoon and Pioneer Bay, Orpheus Is.

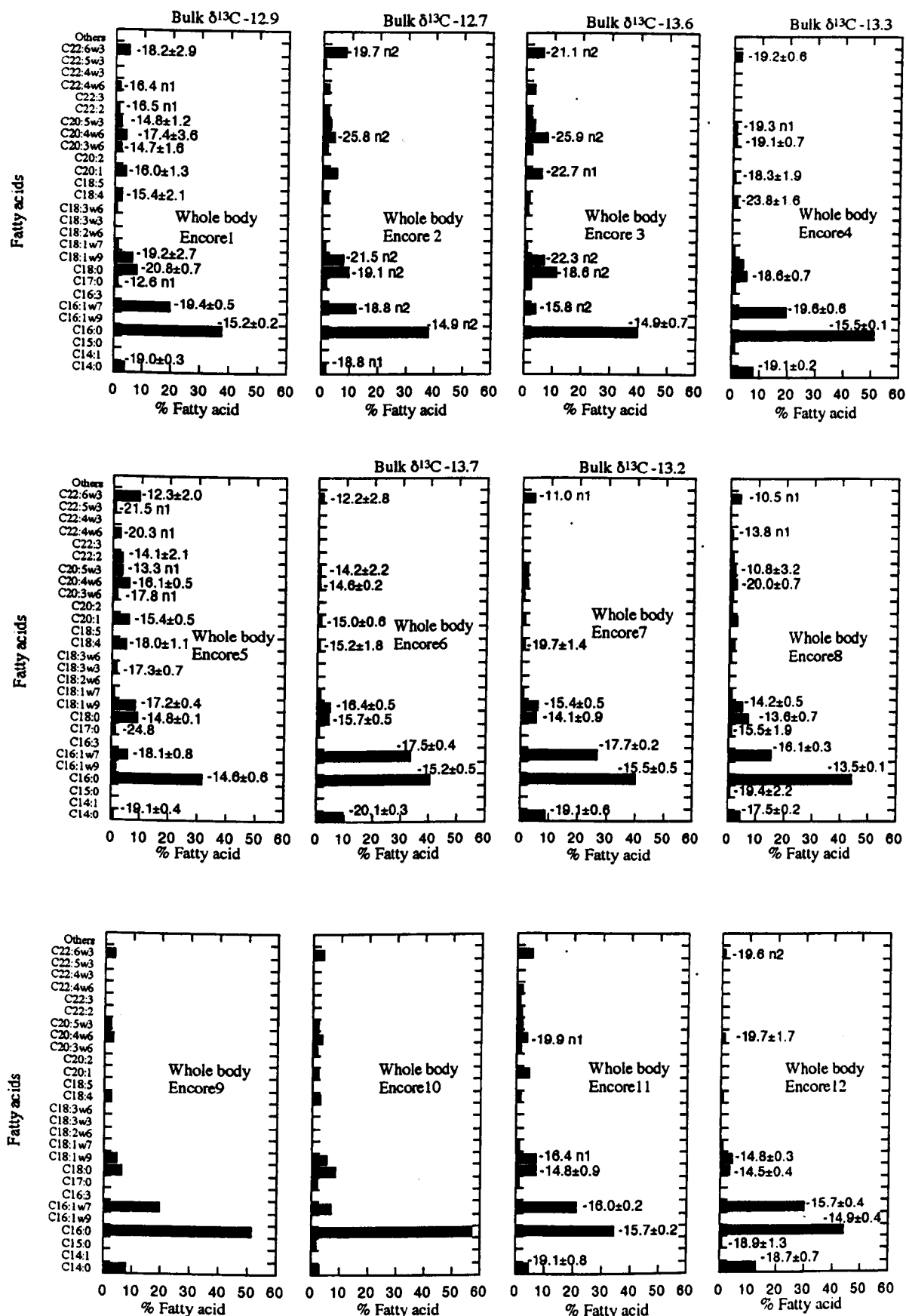


Fig. 3.14 Fatty acid and $\delta^{13}\text{C}$ isotopic profiles for whole body tissues in the symbiotic bivalve *Tridacna maxima*. The twelve individuals (Encore 1-12) were collected from different areas in One Tree Lagoon. Isotopic data are $\delta^{13}\text{C}$ ‰, errors $\pm 1\sigma$. Data tables in appendices.

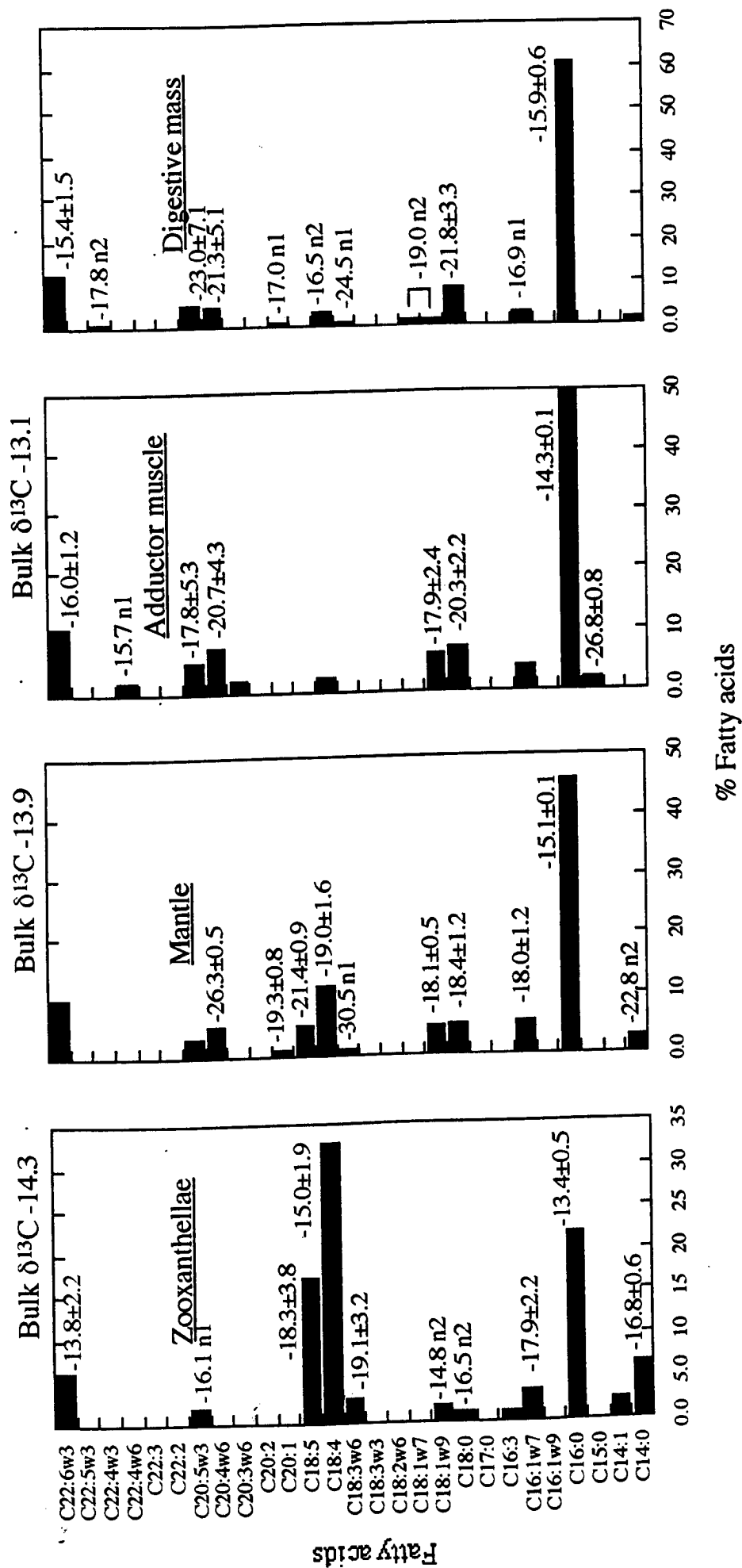


Fig. 3.15 Fatty acid and isotopic profiles for the tissues, mantle, adductor, digestive mass and isolated zooxanthellae in the symbiotic bivalve *T. maxima* (TM1). Values from 1 individual, collected from One Tree Lagoon. (‰, errors 1 σ .) (Data table in appendices)

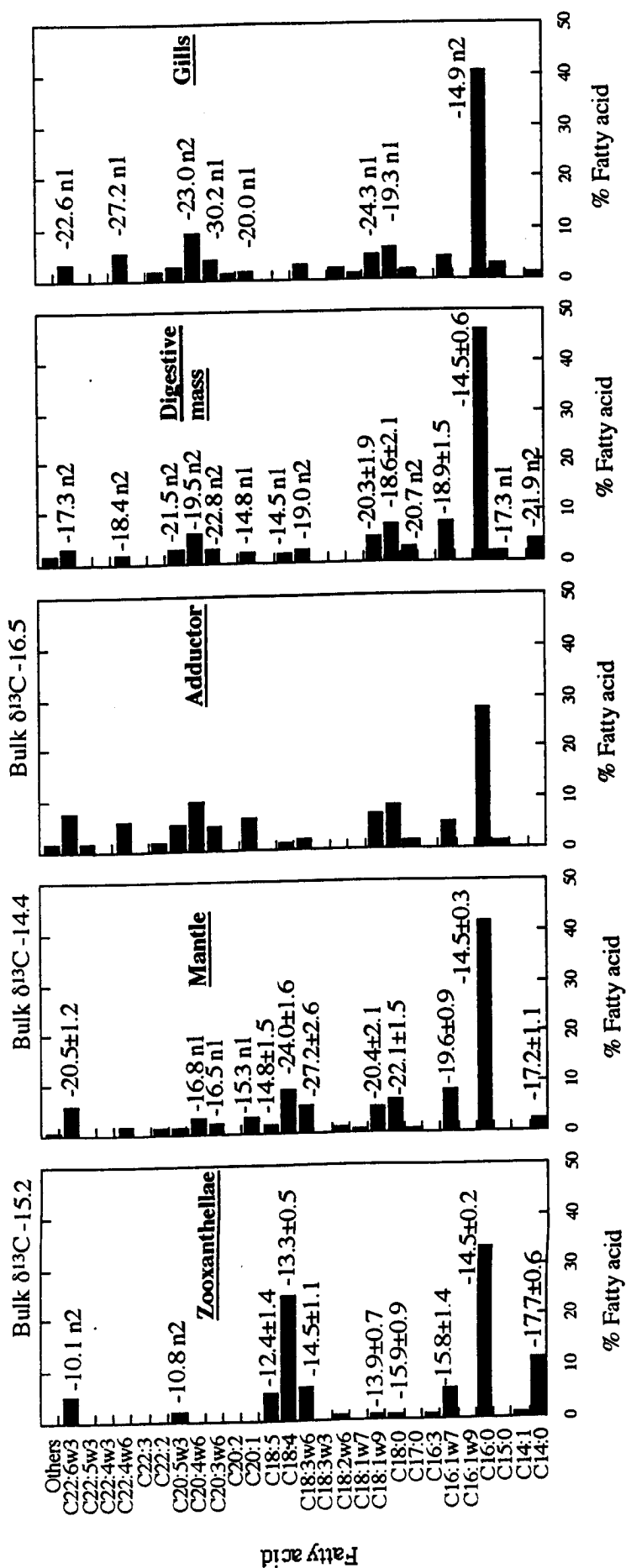


Fig. 3.16 Fatty acid profiles for the tissues mantle, adductor, gills, digestive mass and isolated zooxanthellae in the symbiotic bivalve *Tridacna gigas*, TGI. Values from 1 individual removed from Pioneer Bay, Orpheus Is.

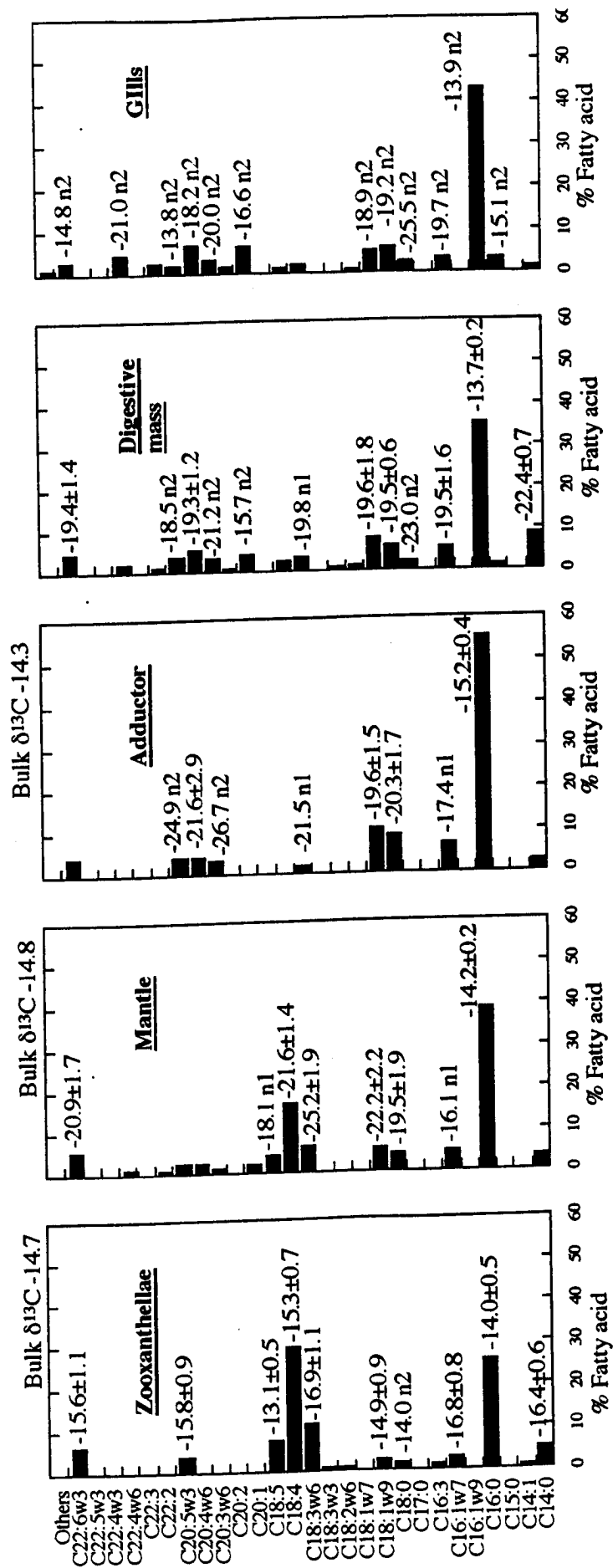


Fig. 3.17 Fatty acid profiles for the tissues, mantle, adductor, digestive mass and gills and isolated zooxanthellae in the symbiotic bivalve *Tridacna gigas* (TG2). Values from 1 individual. The animal, originally from Pioneer Bay, Orpheus Island, was held in the JCU tank for several weeks before sacrificing.)

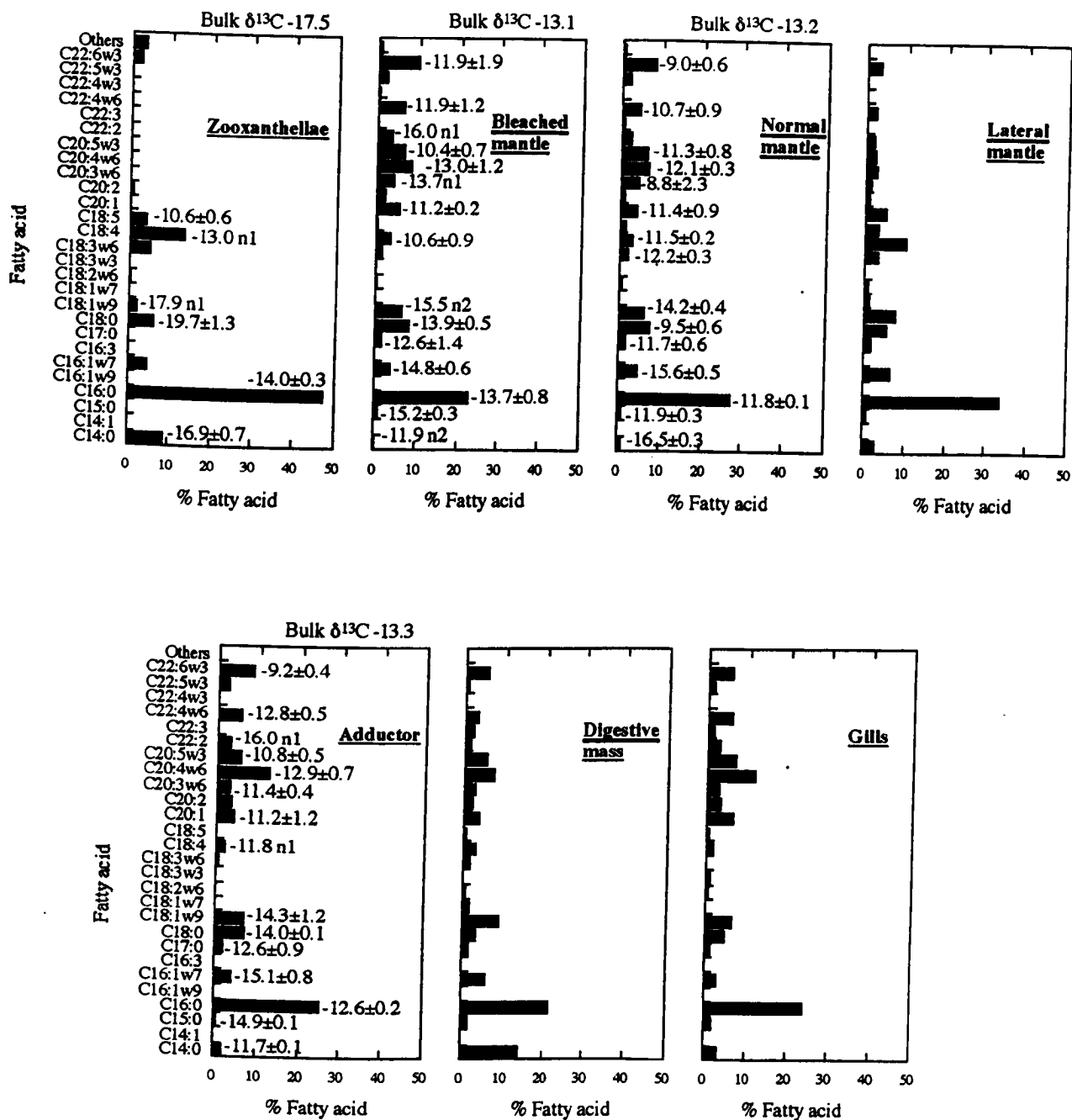


Fig. 3.18 Fatty acid profiles for the tissues, mantle, adductor, digestive mass and gills and isolated zooxanthellae in the symbiotic bivalve *Tridacna gigas* (TG3). Values from 1 partially 'bleached' individual, collected from Pioneer Bay, Orpheus Island. Values are ‰, errors 1 σ .

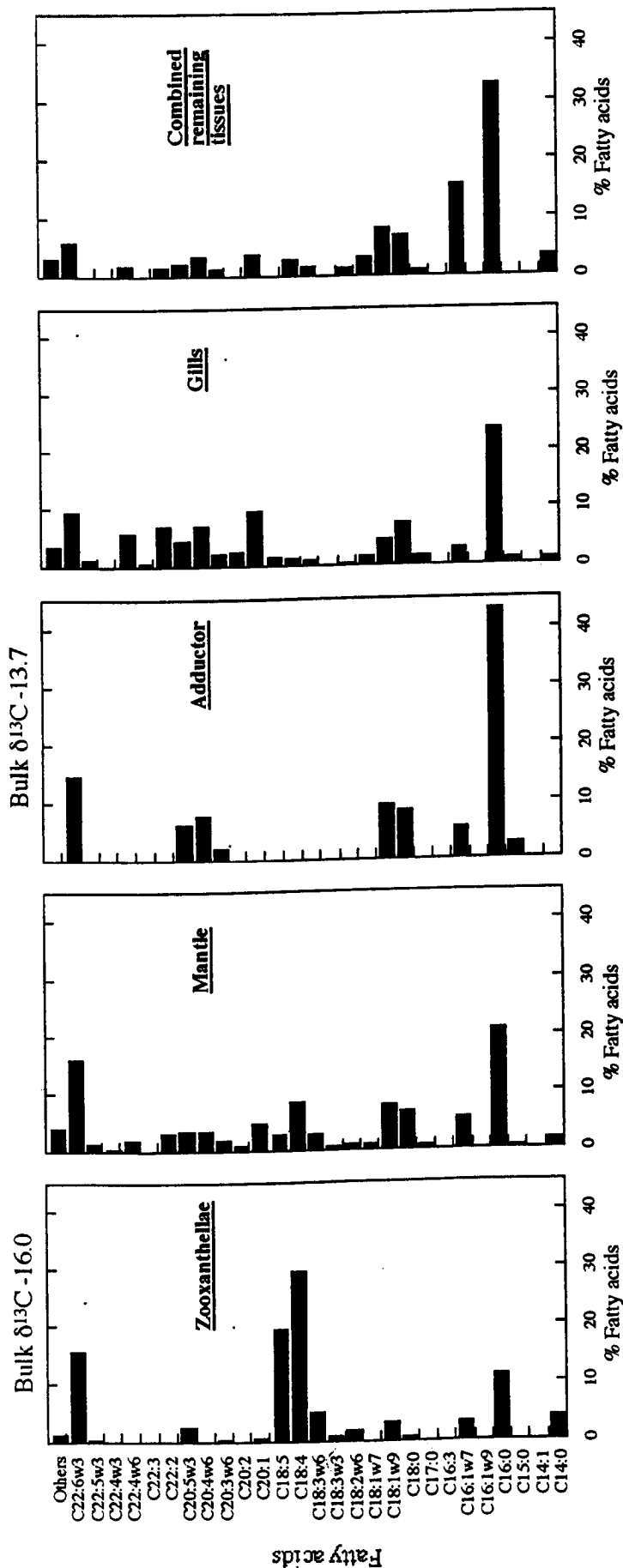


Fig. 3.19 Fatty acid profiles for the tissues, mantle, adductor, digestive mass and gills and isolated zooxanthellae in the symbiotic bivalve *Tridacna squamosa*. Values from 1 individual.

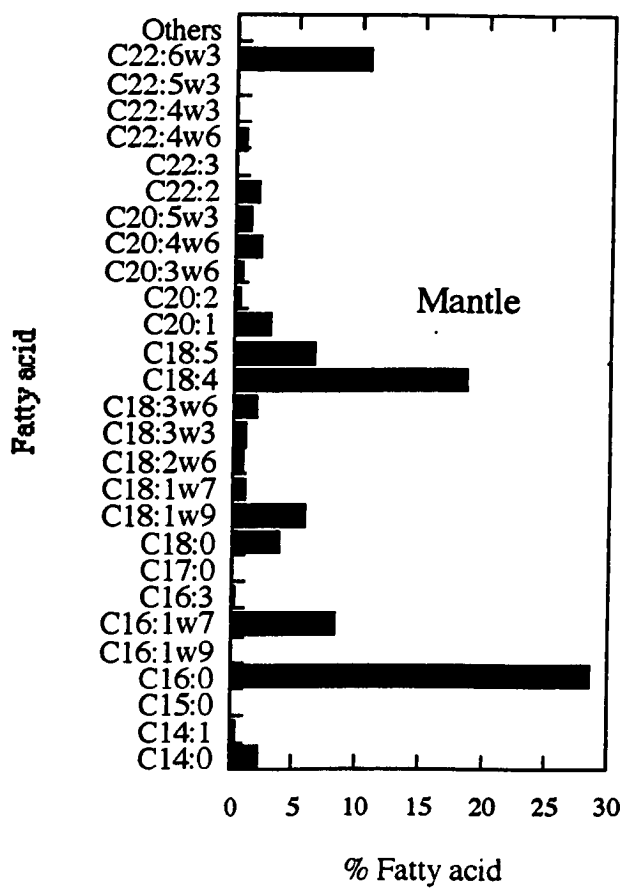


Fig. 3.20 Fatty acid profile for mantle tissue in the symbiotic bivalve *Hippopus hippopus*. Values from 1 individual.

3.7.4 Bulk $\delta^{13}\text{C}$ of tissues isolated from symbiotic bivalves

Bulk tissue $\delta^{13}\text{C}$ values were remarkably similar for all symbiotic bivalve tissues analysed, regardless of species; whole-body (*T. maxima* only) = $-13.2 \pm 0.4\text{‰}$, mantle (*T. gigas*, *T. maxima* and *T. squamosa*) = $-13.7 \pm 0.8\text{‰}$ and muscle (*T. gigas* and *T. maxima*) = $-13.2 \pm 1.4\text{‰}$, Table 3.11.

Table 3.11. Bulk tissue $\delta^{13}\text{C}$ ‰ values for the tissues from symbiotic bivalves, the values are average values and the number of individuals analysed of each species (for each tissue type) are given within parenthesis.

Species	Tissue	Bulk $\delta^{13}\text{C}\text{‰}$
<i>T. gigas</i>	Zooxanthellae	-15.9 ± 1.0 (n=8)
	Mantle	-14.6 ± 1.0 (n=4)
	Muscle	-14.8 ± 1.1 (n=4)
<i>T. gigas albino</i>	Zooxanthellae	-17.5 (n=1)
	Mantle	-13.1 (n=2)
	Muscle	-13.3 (n=1)
<i>T. maxima</i>	Zooxanthellae	-14.3 ± 1.5 (n=10)
	Mantle	-13.4 ± 0.6 (n=2)
	Muscle	-12.6 ± 0.9 (n=7)
	Whole-body	-13.2 ± 0.4 (n=6)
<i>T. squamosus</i>	Zooxanthellae	-16.0 (n=1)
	Mantle	-13.7 (n=1)

The $\delta^{13}\text{C}$ values for whole-body (*T. maxima* only), mantle, and muscle tissues are all slightly ^{13}C -enriched, compared to the isolated zooxanthellae, in the species *T. gigas* (zooxanthellate animals) and *T. maxima* by about 1-2‰. Average $\delta^{13}\text{C}$ values for each tissue, however, are often within error of $\delta^{13}\text{C}_{\text{zooxanthellae}}$ values. Six of seven *T. maxima* exhibit a strong positive correlation between $\delta^{13}\text{C}_{\text{muscle}}$ versus $\delta^{13}\text{C}_{\text{zooxanthellae}}$ ($Y = -1.45 + 0.8X$, $r = 0.986$, Fig. 3.21). One animal (TM9A) appears to have an anomalous $\delta^{13}\text{C}_{\text{zooxanthellae}}$ compared to its contemporaries and does not fit on this regression line. *T. gigas* and *T. squamosa* data are too limited to plot but do not appear to possess a similar correlation.

Terrestrial or aquatic animal whole-body tissue $\delta^{13}\text{C}$ values are usually a fair reflection of that of the animal's diet and are typically ^{13}C -enriched by 0.8-1‰ compared to bulk diet $\delta^{13}\text{C}$ values (DeNiro and Epstein, 1978). Due to their high conversion efficiencies, aquatic invertebrates are expected to be less than 1.0-1.5‰ ^{13}C -enriched compared to dietary $\delta^{13}\text{C}$ (Hayes *et al.*, 1989 and references within). Although POM bulk $\delta^{13}\text{C}$ values have not been analysed for this study, POM and phyto- and zooplankton typically have $\delta^{13}\text{C}$ values of -17 to -26‰ (Table 3.2). Whole-body, mantle and muscle tissue $\delta^{13}\text{C}$ values from symbiotic animals in this study are generally slightly ^{13}C -enriched compared to the range of $\delta^{13}\text{C}$ values reported for POM/phytoplankton/zooplankton. As whole-body, mantle and muscle tissue $\delta^{13}\text{C}$ values from symbiotic animals are within 1-2‰ of $\delta^{13}\text{C}_{\text{zooxanthellae}}$ values in all tissues for all symbiotic species analysed, this is circumstantial evidence that zooxanthellae could be a major contributor in the clam nutrition (DeNiro and Epstein, 1976).

In contrast the albino *T. gigas* has $\delta^{13}\text{C}_{\text{mantle}}$ and $\delta^{13}\text{C}_{\text{muscle}}$ values which are ^{13}C -enriched compared to $\delta^{13}\text{C}_{\text{zooxanthellae}}$ in this animal (by 4.4‰ and 4.2‰ respectively, see table 3.11). Apparently zooxanthellae are not the major contributor to the host nutrition in the albino animal, at least in the recent past, as the isotopic difference between $\delta^{13}\text{C}_{\text{zooxanthellae}}$ and $\delta^{13}\text{C}_{\text{mantle}}$ and $\delta^{13}\text{C}_{\text{muscle}}$ is greater than that assumed from previous studies (DeNiro and Epstein, 1976). It is unknown as to when this animal attained the albino condition, therefore the isotopic composition of body tissues may represent the dietary composition prior to the bacterial infection which atrophied the zooxanthellar tubules. Zooxanthellae also appear to be a lesser contributor to the nutrition of *T. squamosa* in this study as the $\delta^{13}\text{C}_{\text{mantle}}$ in *T. squamosa* is also ^{13}C -enriched compared to $\delta^{13}\text{C}_{\text{zooxanthellae}}$ (by 2.3‰).

3.7.5 CSIA fatty acids isolated from symbiotic bivalves

T. maxima -whole-body tissues

The $\delta^{13}\text{C}$ values of individual fatty acids in whole-body tissues from 10 of the 12 *T. maxima* range from -10.5 to -25.9‰, however the majority of fatty acids have isotopic compositions

between -14 and -20‰ (Fig.3.22). Despite being collected from different areas in the lagoon the $\delta^{13}\text{C}$ values of the 16:0 and 14:0 fatty acids are very similar and average $-15.0 \pm 0.6\text{‰}$ and $-18.9 \pm 0.7\text{‰}$ respectively for all whole-body tissues of this species (average for the ten animals). All other fatty acids show more isotopic variability, presumably reflecting the different localities from which these animals were collected from the lagoon and hence filter-feeding sources (Fig.3.22). No relationship was found between the variation in 16:0/16:1 ω 7 abundance ratios and the isotopic values of those fatty acids. There is no consistent trend of increasing ^{13}C depletion with increasing length of the fatty acids for the nine of the ten animals, which could have been taken as evidence for *de novo* synthesis in the animals. Long chain PUFAs in *T. maxima* whole-body tissues often have as ^{13}C -enriched signatures as the shorter chain saturated fatty acids, although animals Encore 2, 3 and 12 superficially show some ^{13}C -depletion with increasing fatty acid chain length. However, one animal (Encore 6) exhibits increasing ^{13}C -enrichment with increasing chain length and increasing desaturation for the majority of the fatty acids. Individual animal isotopic profiles are quite different in their shape, however 20:4 ω 6 is frequently the most ^{13}C -depleted fatty acid in the profile of all whole-body tissues from ten animals.

Fatty acids isolated from whole-body tissues in *T. maxima* yielded both ^{13}C -enrichments and ^{13}C -depletions relative to $\delta^{13}\text{C}_{16:0}$ in the ten animals. The maximum ^{13}C -enrichment of a fatty acid relative to $\delta^{13}\text{C}_{16:0}$ was 4.5‰, while the maximum ^{13}C -depletion relative to $\delta^{13}\text{C}_{16:0}$ was -10.9‰. The 16:0 fatty acid was generally around 2‰ ^{13}C -depleted compared to $\delta^{13}\text{C}_{\text{whole-body}}$.

T. maxima- mantle, muscle and digestive mass tissues

Individual tissue fatty acid $\delta^{13}\text{C}$ values in *T. maxima* (TM1) ranged from -15.1 to -30.5‰ in mantle, -14.3 to -26.8‰ in muscle and -15.4 to -24.5‰ in digestive mass tissues. The bulk of fatty acids isolated from the individual tissues of *T. maxima* yielded ^{13}C -depleted $\delta^{13}\text{C}$ values compared to $\delta^{13}\text{C}_{16:0}$, with mantle and muscle tissues showing the greatest deviation (Fig.3.23). The isotopic variability of fatty acids in all three *T. maxima* tissues (mantle, muscle and

digestive mass) was greater than that seen in the isotopic variability of the same fatty acids from the isolated zooxanthellae.

T. gigas- mantle, muscle, gills and digestive mass.

$\delta^{13}\text{C}$ values of fatty acids from the individual tissues of the animal TG1 ranged from -14.5 to -27.2‰ in mantle, -14.9 to -30.2‰ in gills and -14.5 to -22.8‰ in digestive mass. $\delta^{13}\text{C}$ values of fatty acids from the individual tissues of the animal TG2 ranged from -14.2 to -25.2‰ in mantle, -15.2 to -26.7‰ in muscle, -13.9 to -25.5 in gills ‰ and -13.7 to -23.0‰ in digestive mass. All fatty acids were ^{13}C -depleted compared to $\delta^{13}\text{C}_{16,0}$ in all tissues with a depletion range of 0-16‰ in TG1 tissues and 0-12‰ in TG2 tissues (Figs. 3.24 and 3.25). The isotopic variability is larger in the mantle, gills and digestive mass tissues isolated from animals TG1 and TG2 than seen in the isotopic variability of isolated zooxanthellar fatty acids from these animals.

The fatty acids isolated from mantle and muscle tissue of the partial albino animal (TG3) gave markedly different $\delta^{13}\text{C}$ values compared with the same fatty acids from its fully zooxanthellate contemporaries. Fatty acid $\delta^{13}\text{C}$ values ranged from -10.4 to -16.0‰ in bleached mantle, -8.8 to -15.6‰ in 'normal' mantle and -9.2 to -16.0‰ in muscle tissues in the animal TG3. Approximately 50% of the fatty acids in TG3 mantle and muscle were ^{13}C -enriched compared to $\delta^{13}\text{C}_{16,0}$ (Fig. 3.26). The difference in isotopic composition of the fatty acids in TG3 mantle and muscle relative to the $\delta^{13}\text{C}_{16,0}$ ranged from around +4 to -5‰. This is quite different to the other fully zooxanthellate animals and must presumably be related in some way to the partial bleaching of this animal. The numbers of zooxanthellae are much reduced in this animal, yet the animal is still filter feeding. One would thus expect the isotopic values of the fatty acids to be biased towards that of POM and *de novo* synthesis by the clam. Yet the POM fatty acids analysed in this study were all more ^{13}C -depleted than any of the fatty acid $\delta^{13}\text{C}$ values seen in the mantle and muscle tissues of TG3, see section 3.5.3 and Fig. 3.18.

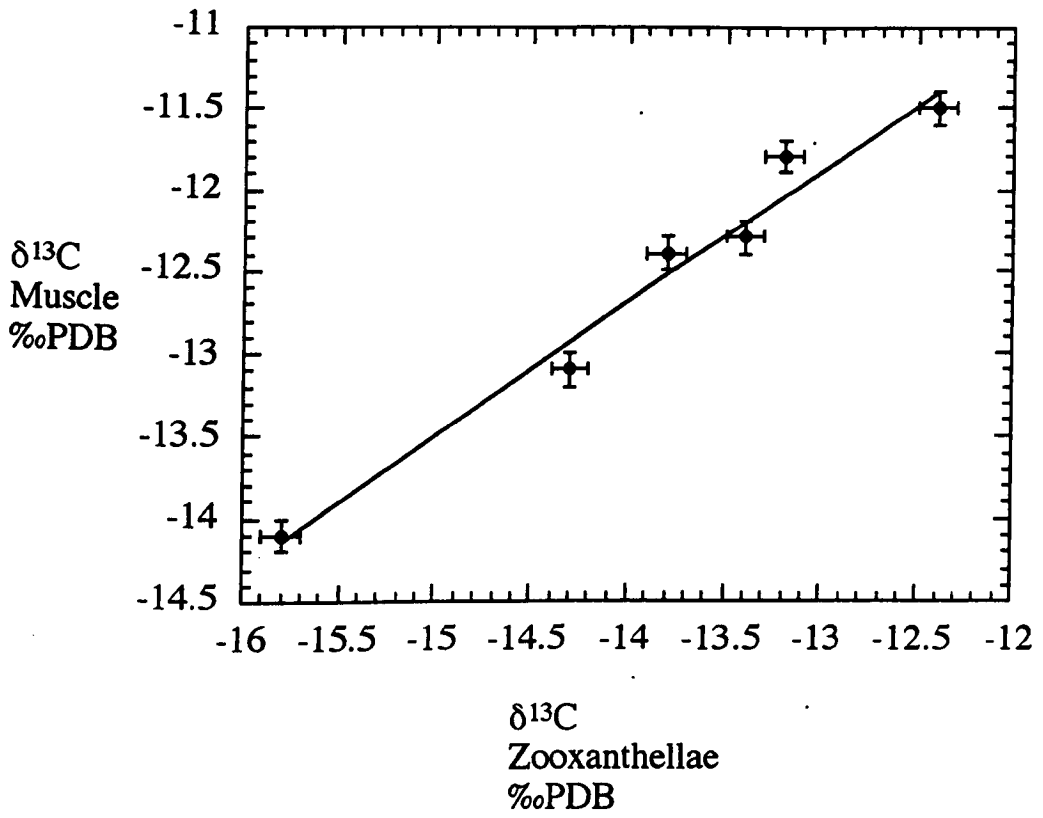


Fig. 3.21 $\delta^{13}\text{C}$ compositions of muscle versus zooxanthellae isolated from *T. maxima*.

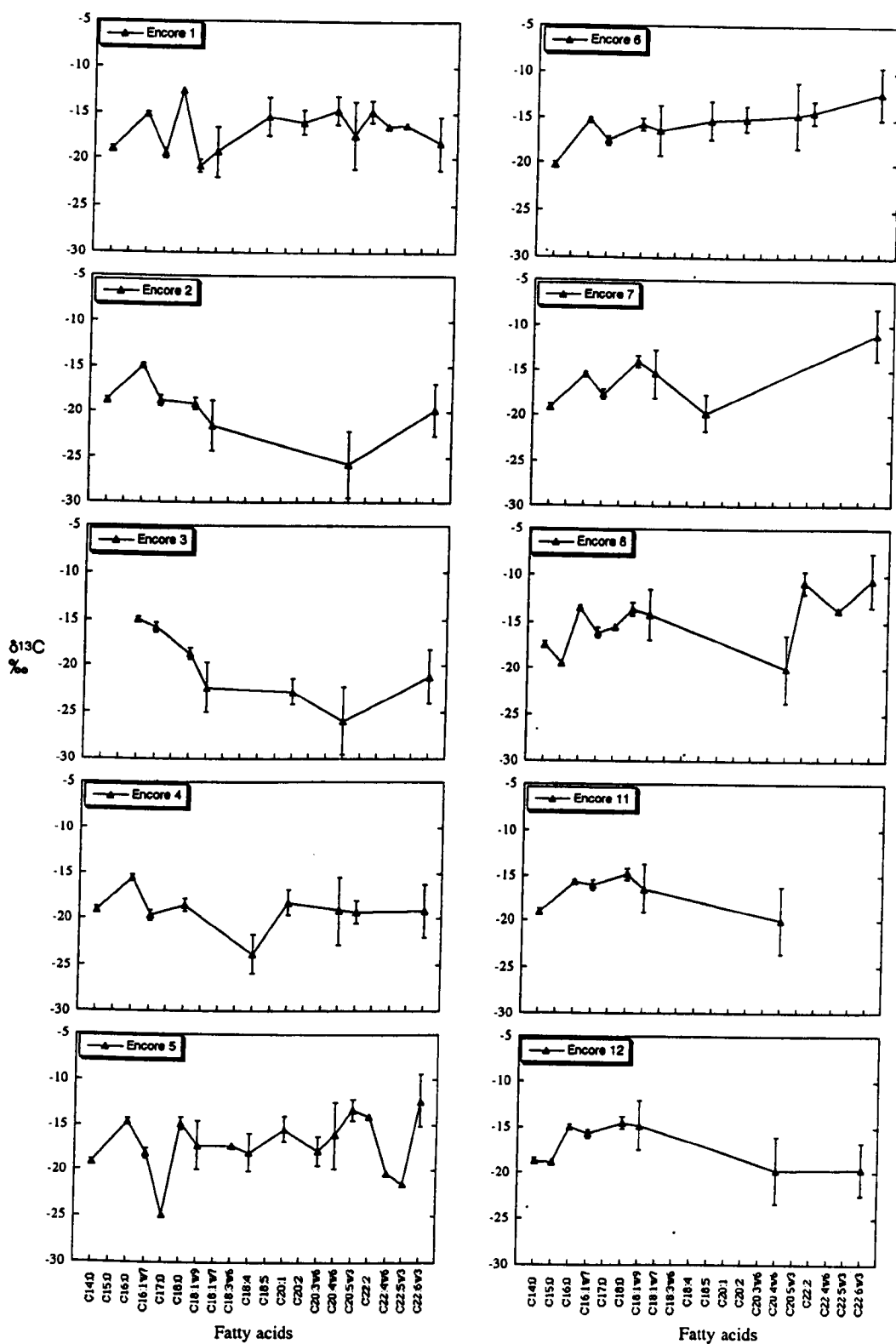


Fig. 3.22 $\delta^{13}\text{C}$ isotopic profiles of fatty acids isolated from whole body tissues in *T. maxima*, collected from OTI

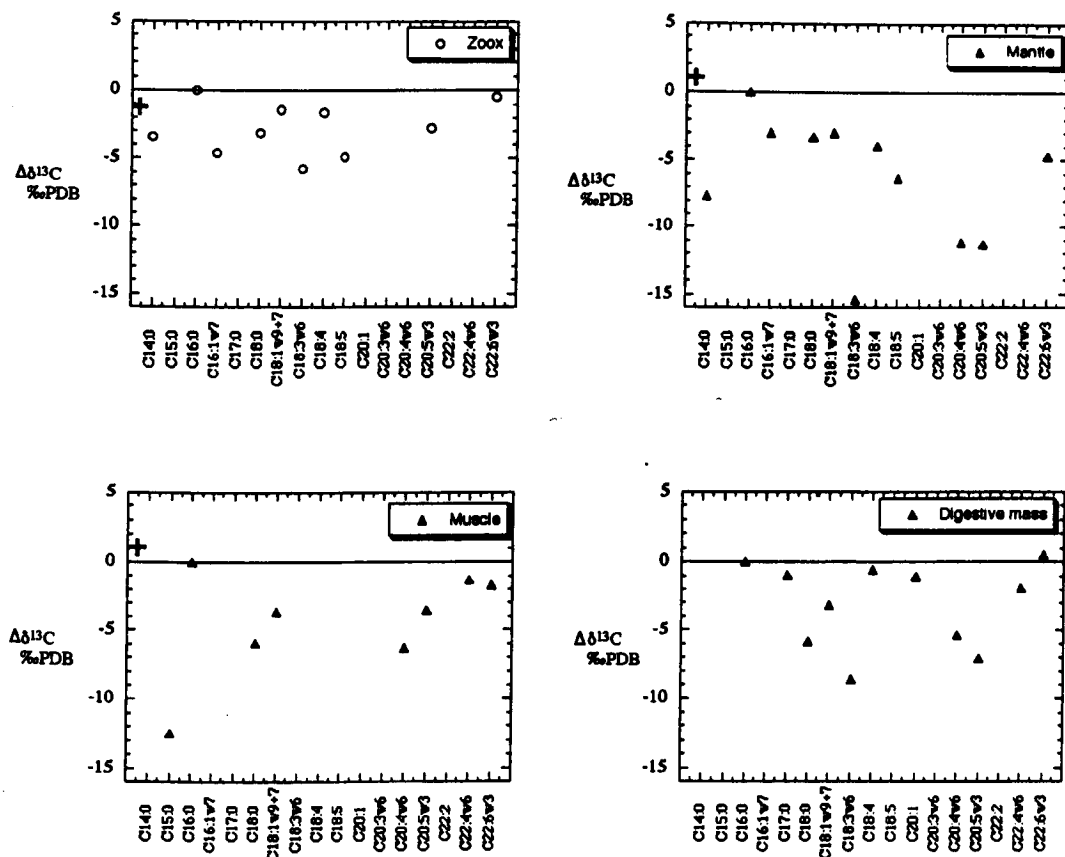


Fig. 3.23 Isotopic compositions of fatty acids in tissues of *T. maxima* relative to palmitate ($\delta^{13}\text{C} \text{ ‰}$).

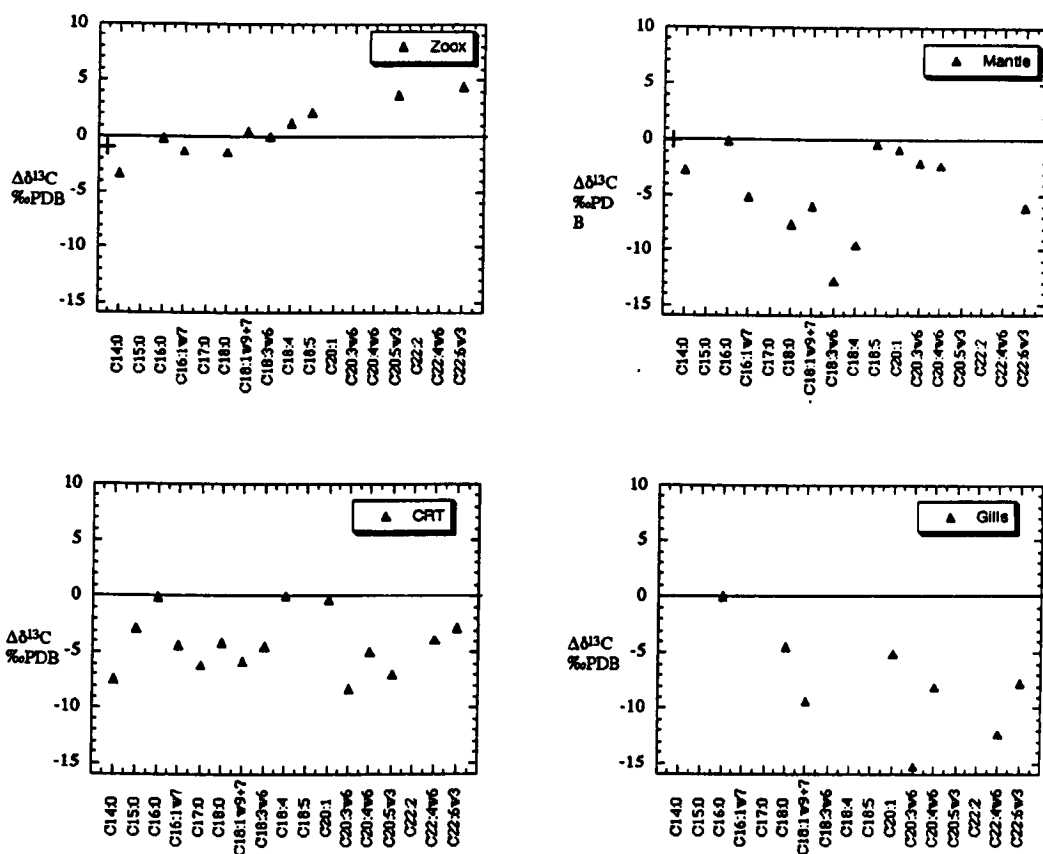


Fig. 3.24 Isotopic compositions of fatty acids in the tissues of *T. gigas* (TG1) relative to palmitate ($\delta^{13}\text{C}$ ‰).

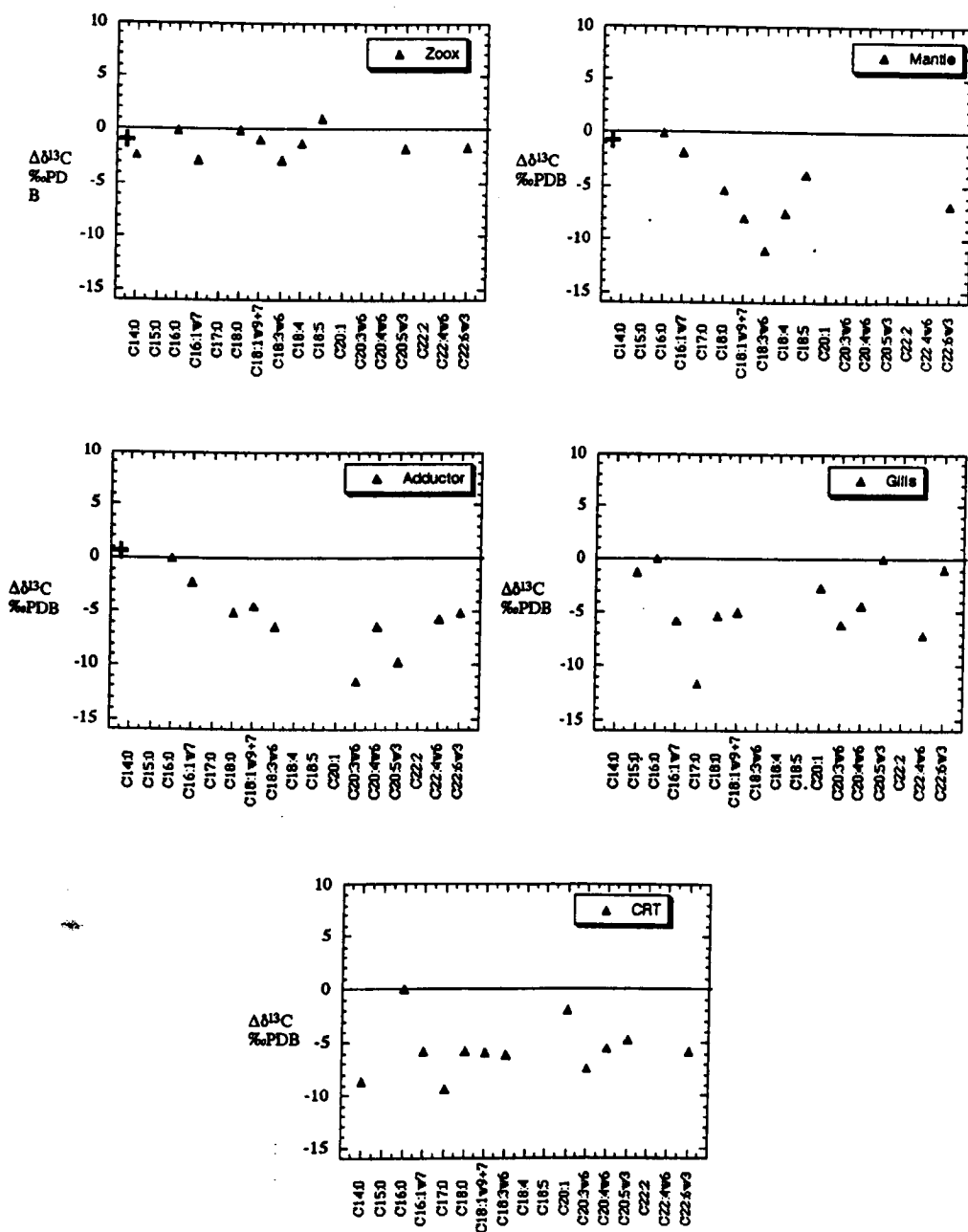


Fig. 3.25 Isotopic compositions of fatty acids in tissues of *T. gigas* (TG2) relative to palmitate ($\delta^{13}\text{C} \text{ ‰}$)

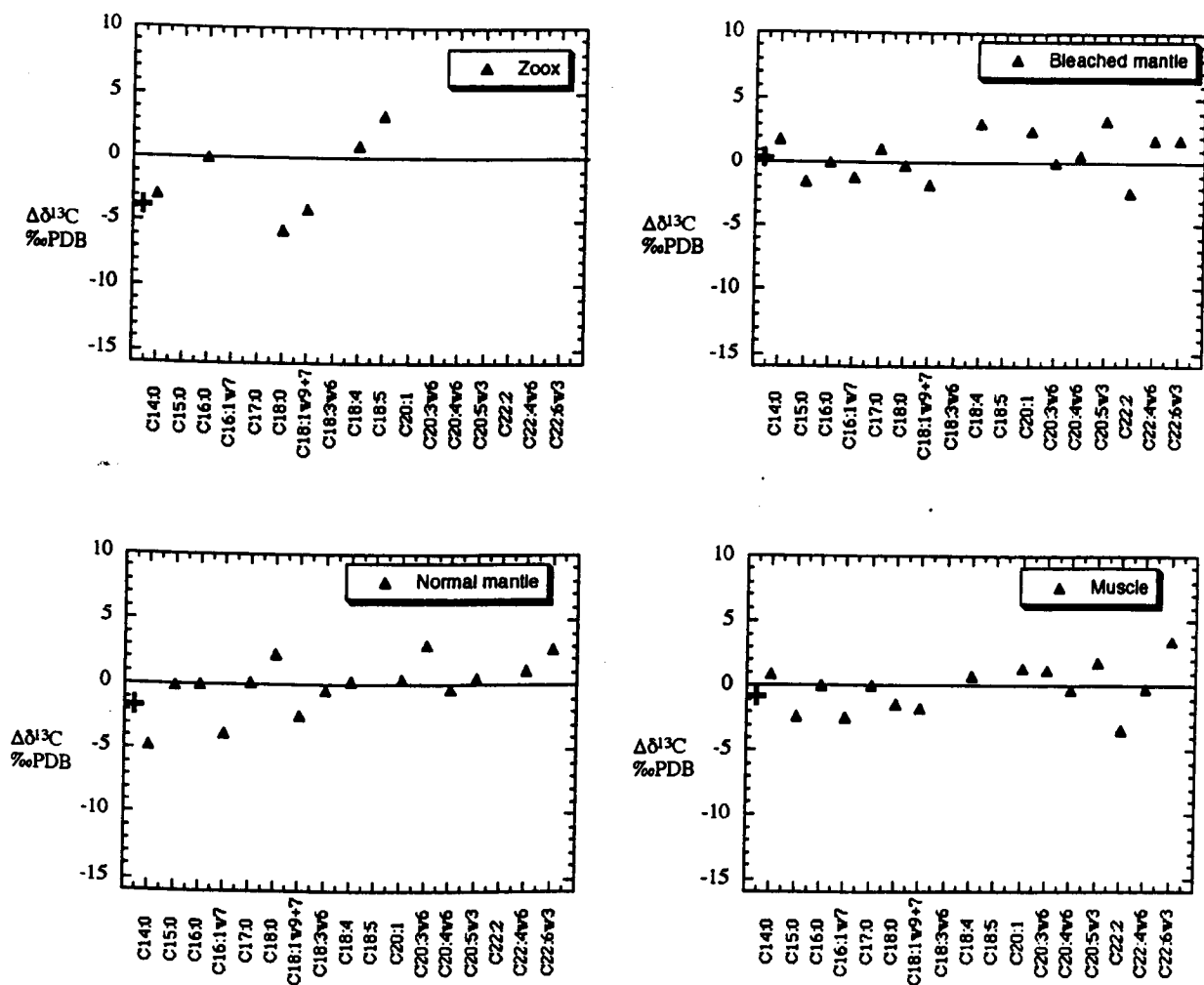


Fig. 3.26 Isotopic compositions of fatty acids in tissues of the partial albino *T. gigas* (TG3) relative to palmitate $\delta^{13}\text{C}$.

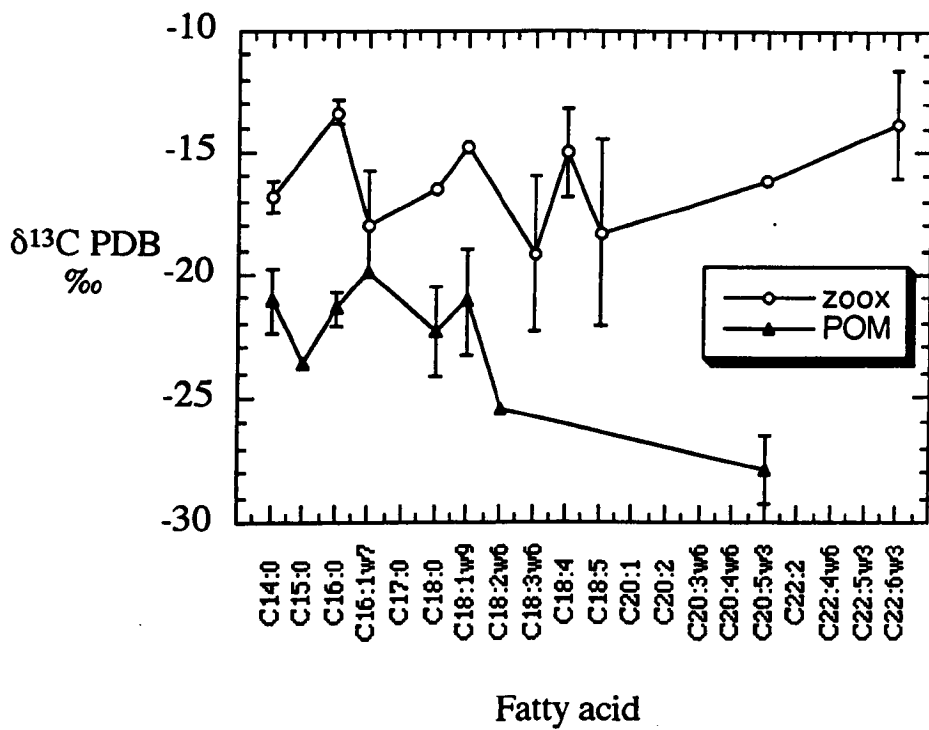


Fig. 3.27 $\delta^{13}\text{C}$ isotopic compositions of individual fatty acids isolated from zooxanthellae, isolated from *T. maxima* compared with those of POM taken from the same collected site. $\delta^{13}\text{C}$ compositions are average values from 10 POM samplings (OT lagoon) and zooxanthellae from one animal, TM1. (errors 1σ)

3.8 IMPLICATION OF FATTY ACID RESULTS TO THE SYMBIOSES

The data for POM collected from One Tree Lagoon, Pioneer Bay (Orpheus) and from the JCU aquarium represent time integrated values over a very short time period. Such a snapshot sampling reflects the dietary POM over the recent past and may not be indicative of long-term dietary fatty acid concentrations or isotopic compositions for filter feeding animals. There is a lack of published carbonisotopic data on individual fatty acids extracted from POM. Therefore these data are the only reasonable estimate of the range of the fatty acids and $\delta^{13}\text{C}$ values of the individual fatty acids that are available to filter feeders in the locations, and have been used in section 3.8 to infer possible nutritional sources for *T. maxima* and *T. gigas*.

3.8.1 *T. maxima*

Whole-body tissues, POM and zooxanthellae

The major fatty acids found in the zooxanthellae isolated from *T. maxima* were 14:0, 16:0, 16:1 ω 7, 18:4, 18:5, 20:5 ω 3 and 22:6 ω 3. These same fatty acids were also present in *T. maxima* whole-body tissues. Zooxanthellae were present extensively in *T. maxima* tissues, especially in the mantle, and because of the nature of the association it is not physically possible to separate the mantle tissues from the zooxanthellae so it is to be expected that similar fatty acids would be found in the whole-body extract. However, the abundances of the fatty acids are different: 16:0, 18:4 and 18:5 are the most abundant fatty acids in zooxanthellae, and although 16:0 is the most abundant in whole-body tissues, both 18:4 and 18:5 (when present) are at much lower abundances. Thus from abundance data alone, likely candidates for translocated fatty acids would appear to be 14:0, 16:0, 16:1 ω 7, 18:0 and 18:1 ω 9.

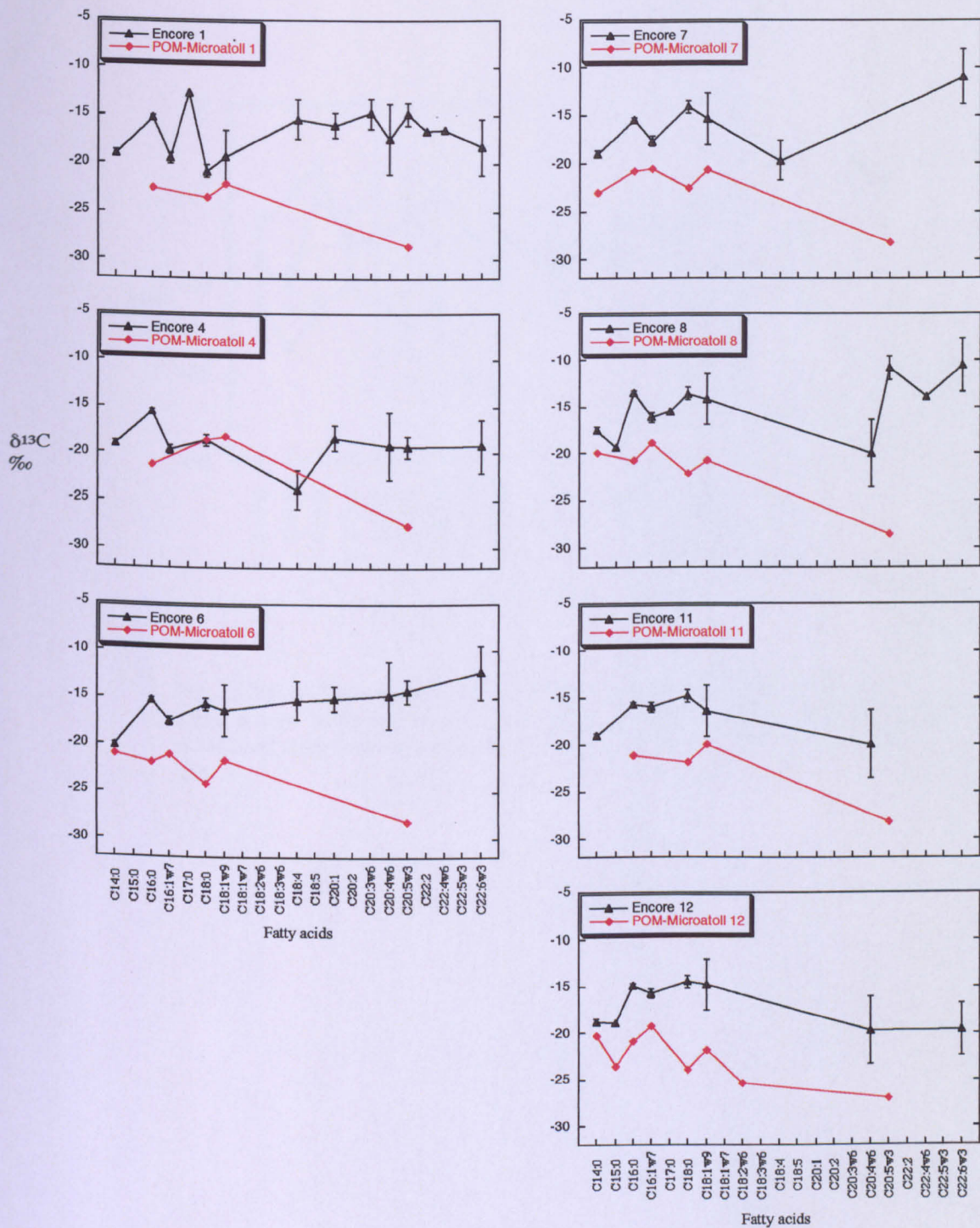


Fig. 3.28 $\delta^{13}\text{C}$ isotopic profiles of fatty acids isolated from whole body tissues in *T. maxima* and POM, collected from OTI.

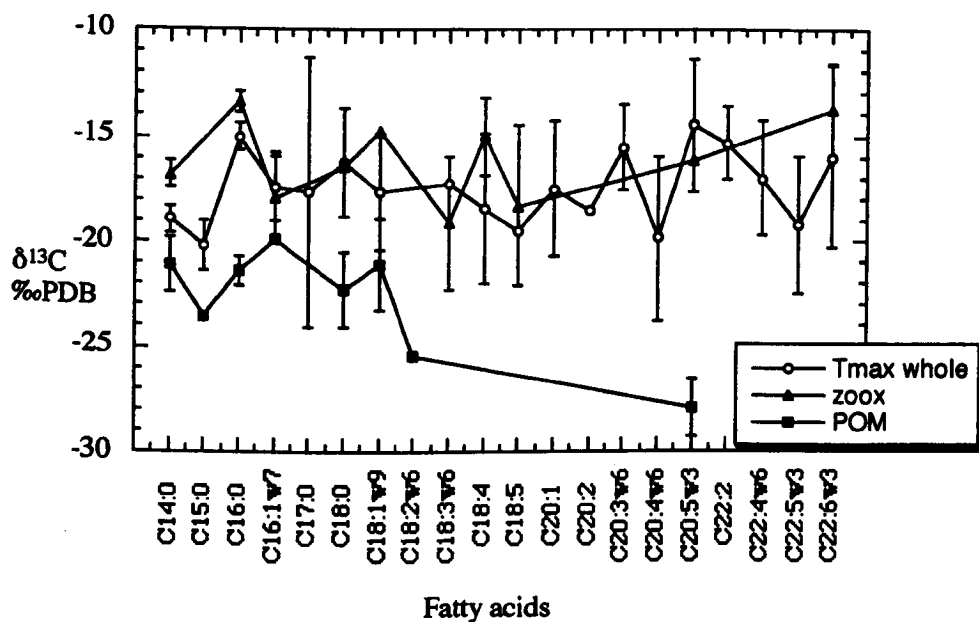


Fig. 3.29a: $\delta^{13}\text{C}$ isotopic compositions of individual fatty acids isolated from whole body tissues of *T. maxima* (average of 12 individuals), compared with those acids in isolated zooxanthellae and POM from the site

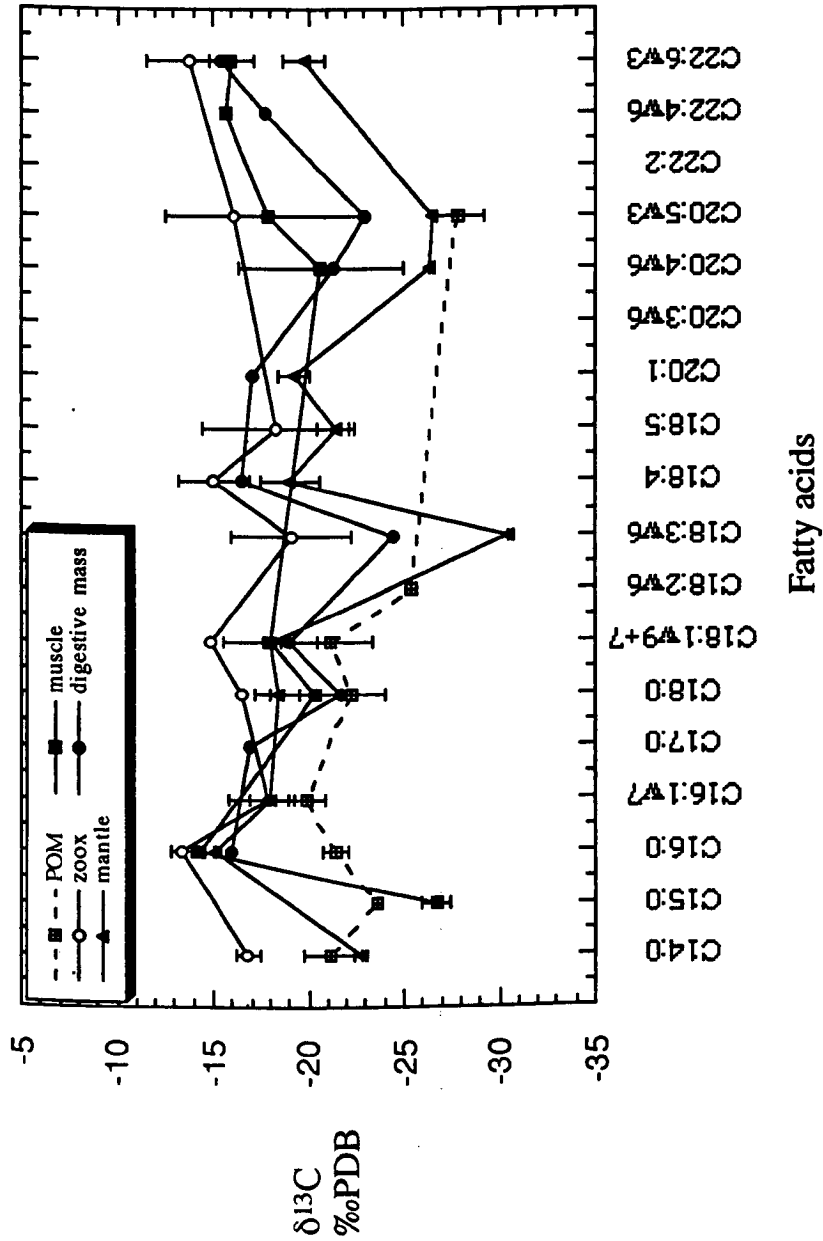


Fig. 3.29b $\delta^{13}\text{C}$ isotopic compositions of individual fatty acids isolated from *T. maxima* compared with those from POM, collected from the same site. (Shaded area emphasises the isotopic composition range of POM from the site). Zoxanthellae, mantle, muscle and digestive mass from animal TM1, collected from OT lagoon (errors 1σ)

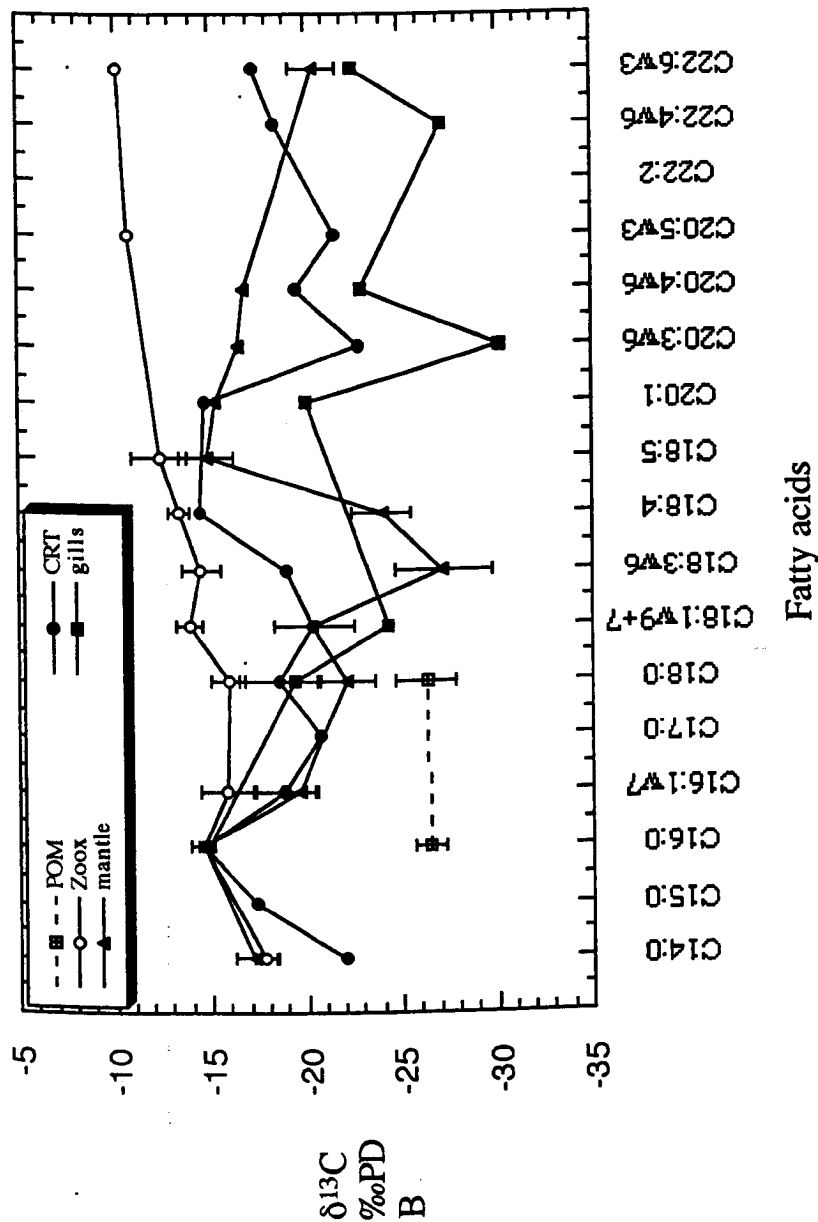


Fig. 3.30 $\delta^{13}\text{C}$ isotopic compositions of individual fatty acids isolated from *T. gigas* compared with those from POM collected from the same site. Zooxanthellae, mantle, gills and digestive mass from animal TG1, collected from Pioneer Bay, Orpheus Is. (errors 1σ)

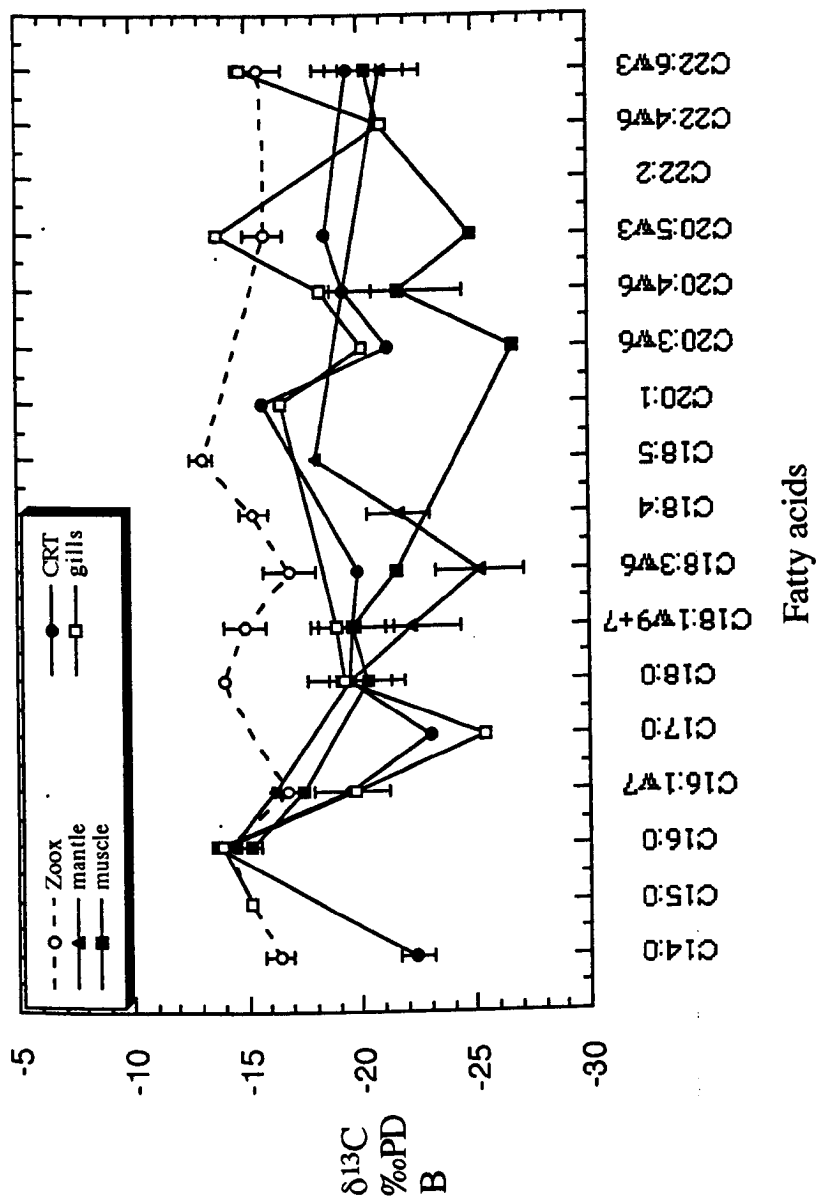
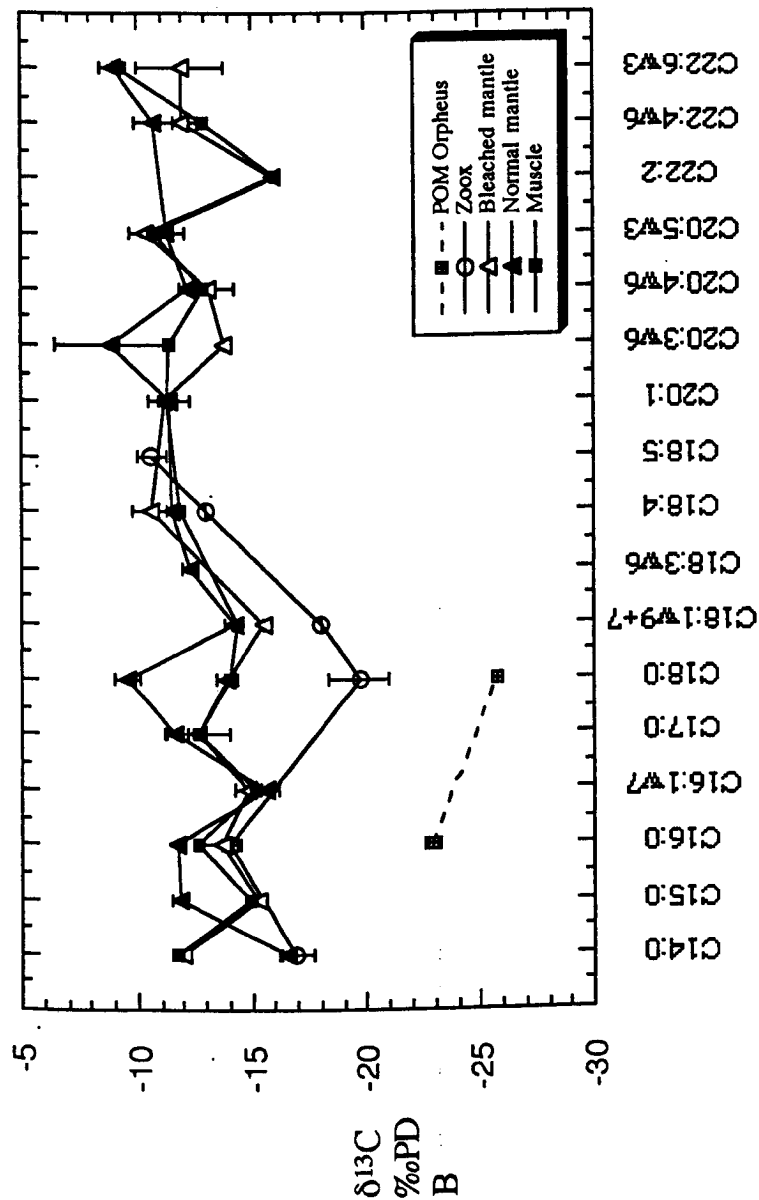


Fig. 3.31 $\delta^{13}\text{C}$ isotopic compositions of individual fatty acids isolated from *T. gigas*. Zooxanthellae, mantle, muscle, gills and digestive mass from animal TG2, initially collected from Pioneer Bay, Orpheus Is but held in JCU aquarium for several weeks prior to sacrificing.



Fatty acids

Fig. 3.32 $\delta^{13}\text{C}$ isotopic compositions of tissues and zooxanthellae isolated from a partial albino *T. gigas*, TG3, collected from Pioneer Bay, Orpheus Is compared with those from Pom collected from the same site.

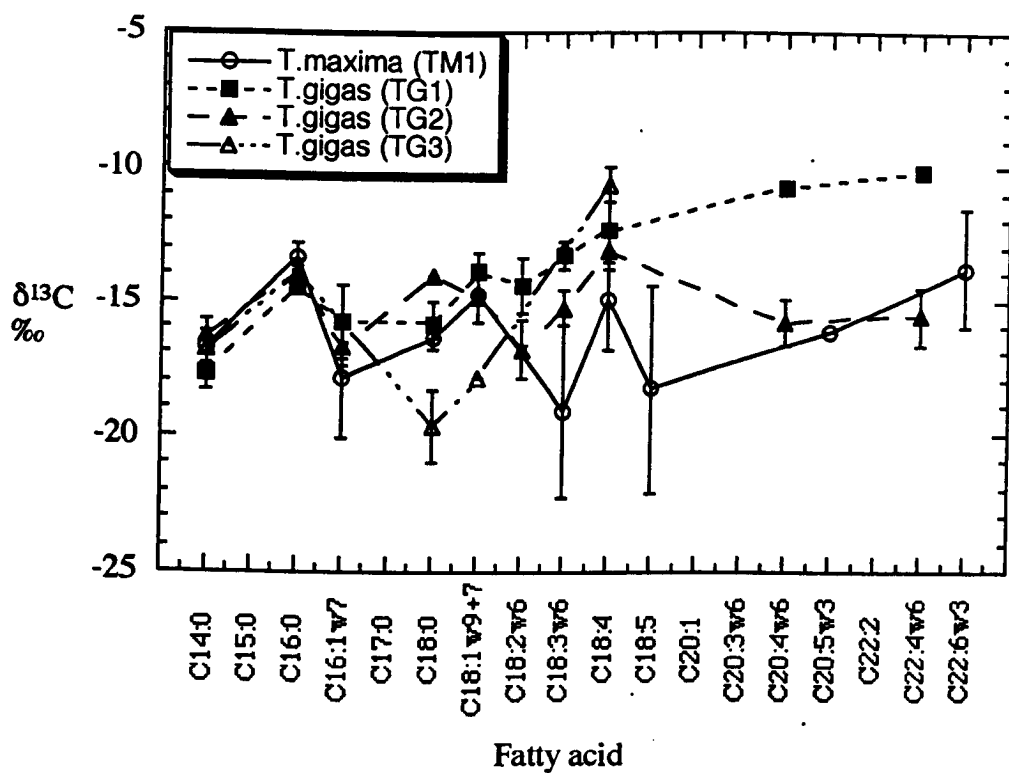


Fig. 3.33 $\delta^{13}\text{C}$ isotopic compositions of individual fatty acids isolated from zooxanthellae in the species *T. gigas* and *T. maxima*

The isotopic compositions of the fatty acids obtained from isolated *T. maxima* zooxanthellae were ^{13}C -enriched compared to fatty acids isolated from POM taken from the same sampling site (Fig.3.27). Comparison of the fatty acids isolated from whole-body tissues with those from POM also reveals that the clam fatty acids are generally ^{13}C -enriched compared to those from POM at 7 sites (Fig. 3.28). Whole-body fatty acid $\delta^{13}\text{C}$ values are very similar to zooxanthellae fatty acid $\delta^{13}\text{C}$ values for many of the fatty acids (Fig.3.29a). In particular the whole-body fatty acids 16:1 ω 7 and 18:0 are within 0.5‰ of $\delta^{13}\text{C}$ values of those same fatty acids in zooxanthellae. Furthermore the fatty acids 16:0, 18:3 ω 6 18:5, 20:5 ω 3 in whole-body extracts are within 1.6, 1.9, 1.2 and 1.6‰ respectively of the same fatty acids in zooxanthellae. It is difficult to attribute fatty acid sources from these data alone. If both the average $\delta^{13}\text{C}$ fatty acid values of POM and zooxanthellae are plotted with *T. maxima* whole-body fatty acid $\delta^{13}\text{C}$ values, it is apparent that several whole-body fatty acids are more similar to zooxanthellae fatty acid $\delta^{13}\text{C}$ values, than they are to POM fatty acid $\delta^{13}\text{C}$ values (Fig.3.29a). These isotopically similar fatty acids are apparently mainly zooxanthellar in origin rather than sourced from filter feeding.

The fatty acids 16:0, 16:1 ω 7 and 18:0 are also the most likely first products of photosynthesis in the zooxanthellae during fatty acid synthesis. Fatty acid synthesis in all eukaryotes involves the repetitive condensation of two-carbon units (derived from malonyl CoA) with a growing acyl chain, followed by the reduction of the condensation product to typically form short chain fatty acids. Fatty acid synthesis is often referred to as palmitate synthesis because 16:0 is the preponderant product. There are two types of fatty acid synthetases in different organisms (Type I and Type II) and dinoflagellates possess a Type I synthetase (Gurr and Harwood, 1991). The typical products of Type I synthetases are free 16:0 and C16 to C24-CoA and under most conditions 16:0 and 18:0 are the main products of biosynthesis in plastids or chloroplasts (Gurr and Harwood, 1991). The fatty acid $\delta^{13}\text{C}_{14,0}$ in whole-body tissues of *T. maxima* is intermediate between the $\delta^{13}\text{C}$ composition of 14:0 fatty acids of isolated

zooxanthellae and POM, which may reflect a contribution from both zooxanthellae and POM sources for this fatty acid. It is intriguing that $\delta^{13}\text{C}_{14:0}$ is always more ^{13}C depleted than $\delta^{13}\text{C}_{16:0}$ in zooxanthellae and in the whole-body fatty acids of *T. maxima*. If 16:0 had been synthesised from the elongation of 14:0 it would be enriched in ^{13}C compared to 16:0 (Schouten *et al.*, 1998). This anomaly was also found in the various tissues of the *T. gigas* animals TG1 and TG2 (section 3.8.2) and may point to a parallel fatty acid biosynthesis, 14:0 and 16:0 being synthesised from different carbon sources or possibly produced by different fatty acid synthetases favouring different chain terminations i.e. different chain lengths. ^{13}C -depleted 14:0 fatty acids compared to $\delta^{13}\text{C}_{16:0}$ have been reported before in the study of microbial lipids (Abraham *et al.*, 1998) The fatty acids 15:0 and 17:0 were not found in zooxanthellae analysed for this study and could be bacterial in origin (Linley and Koop, 1986). Elongation of the fatty acids 14:0 or 16:0, to provide 15:0 and 17:0, cannot be ruled out, as both algae and animals possess the necessary enzymes. The 15:0 fatty acid $\delta^{13}\text{C}$ is ^{13}C -depleted compared to 14:0 as is the 17:0 compared to 16:0 which in itself may suggest elongation, however the ^{13}C -depletions seen in 15:0 and 17:0 are larger than would be expected from simple transformations from 14:0 and 16:0 fatty acids (Schouten *et al* 1998).

Attributing sources for PUFAs in *T. maxima* is complicated due to the paucity of PUFAs in the POM samples. It is unlikely that PUFAs other than 20:5 ω 3 are absent at One Tree Lagoon. Algal compositions will change seasonally and in response to the nutritional status of the water column. However, the fatty acids 18:3 ω 6, 18:5 and 20:5 ω 3 in whole tissues are within 2‰ of zooxanthellar isotopic compositions which may indicate direct translocation or simple transformations from shorter chain fatty acids. The 20:5 ω 3 isolated from POM for this site in particular is some 10‰ different to that found in clam tissues and is therefore unlikely to be sourced from POM.

In the case of *T. maxima* whole-body tissues, some zooxanthellar fatty acids are apparently incorporated into host tissues. These fatty acids are presumably translocated, and are used

directly, without further modification by the host. Although fatty acids may be subsequently elongated or desaturated by the host it is not immediately apparent from the data that it is occurring in *T. maxima*, as several of the ^{13}C -depletions relative to short chain fatty acid isotopic compositions were larger than expected from isotopic effects due to simple elongation and desaturation transformations (Schouten *et al.*, 1998). Positional isomers were established for many of the fatty acids in this study; however suitable standards were not available to establish all isomers. Elongation of the zooxanthellar fatty acids 16:1 ω 7 and 18:1 ω 9 could provide the fatty acids 20:1, 20:2 and 22:2. Three isomers were found for 20:1, however, they could not be resolved chromatographically for isotopic analysis, therefore the $\delta^{13}\text{C}$ composition reported represents a mix of the 3 isomers. Elongation of 18:1 to 20:1 and 22:2 fatty acids has been reported before in bivalves (Langdon and Waldock, 1981). Zooxanthellar 18:3 ω 6 could also be elongated to form 20:3 ω 6, subsequently desaturated to 20:4 ω 6 and followed by elongation to produce 22:4 ω 6. All three of these fatty acids were found in the whole-body clam tissues. Although the 18:4 and 18:5 positional isomers are unknown, their likely form is ω 3, as previous studies have reported ω 3 forms of these fatty acids from dinoflagellates, and not ω 6 (Harrington *et al.*, 1970). The reason for the low levels of 18:3 ω 3 in both zooxanthellae and clam whole-body tissues may be because this fatty acid is transformed, as soon as it is synthesised, to produce the fatty acids 18:4 ω 3 > 18:5 ω 3 > 20:5 ω 3 > 22:5 ω 3 and 22:6 ω 3. However the Δ 6, Δ 5 and Δ 4 desaturases in bivalves are often absent or rate-limiting which would make this particular route less likely (Langdon and Waldock, 1981). Both 18:2 ω 6 and 18:3 ω 3 were absent in significant amounts and their low abundances may be a reflection of the rapid usage of these fatty acids to produce longer PUFAs. It is noteworthy that some PUFAs from zooxanthellae and symbiotic bivalve whole-body tissues are ^{13}C enriched compared to the saturated fatty acids, which would presumably be their short chain precursors for chain transformations. In order to focus in on the tracing of translocation of fatty acids in *T. maxima* and *T. gigas* it is necessary to look in more detail at the individual tissues in the symbioses.

T. maxima – mantle, muscle and digestive mass tissues

Fatty acids from mantle tissue contained all the predominant acids found in isolated zooxanthellae with relatively high levels of 18:4 and 18:5 compared to muscle and digestive mass tissues. Adductor muscle in *T. maxima* had the fatty acids 16:0, 16:1 ω 7 18:0, 18:1 ω 9, 20:4 ω 3, 20:5 ω 3 and 22:6 ω 3 as the most predominant fatty acids, all of which occur in isolated zooxanthellae. Digestive mass tissues in *T. maxima* yielded a fatty acid profile that was dominated by 16:0. In comparing the four isotopic profiles of tissues and zooxanthellae isolated from *T. maxima* (Fig. 3.15 and 3.29) it would appear that adductor muscle bears more resemblance to the digestive mass fatty acid profile than to that of the isolated zooxanthellae. Mantle and muscle tissues isolated from *T. maxima* had $\delta^{13}\text{C}_{16:0}$ values that were within 1.7‰ and 0.9‰ of zooxanthellar $\delta^{13}\text{C}_{16:0}$ respectively (Figs. 3.15 and 3.29b). As muscle does not contain a zooxanthellar component this is strong evidence for direct translocation of zooxanthellar 16:0 to this tissue. In contrast the $\delta^{13}\text{C}_{18:0}$ and $\delta^{13}\text{C}_{18:1\omega9+7}$ (these monounsaturated acids could not be resolved chromatographically for these samples) from *T. maxima* muscle and mantle tissues were intermediate between the zooxanthellar and POM values for these fatty acids. Although the $\delta^{13}\text{C}_{18:0}$ in ten whole-body tissues from *T. maxima* (Encore samples) suggested 18:0 was a major zooxanthellar translocated fatty acid, it is apparent for animal TM1 that this is not the case, and that 18:0 is sourced either from a combination of both zooxanthellar and filter feeding sources or is produced *de novo* in the clam. The difference between $\delta^{13}\text{C}_{16:0}$ and $\delta^{13}\text{C}_{18:0}$ is too large to be an isotopic effect due to elongation of the fatty acid 16:0. Although 16:1 ω 7 was found to be a possible translocated fatty acid in whole-body tissue from *T. maxima*, it is difficult to attribute the fatty acid 16:1 ω 7 found in the mantle tissues of *T. maxima* to either a zooxanthellar or POM source, as the $\delta^{13}\text{C}_{16:1\omega7}$ isotopic signatures are very similar, the mantle $\delta^{13}\text{C}_{16:1\omega7}$ is within 1‰ of the zooxanthellae $\delta^{13}\text{C}_{16:1\omega7}$ and within 2‰ of the $\delta^{13}\text{C}_{16:1\omega7}$ in POM. The fatty acid 20:5 ω 3 is also a possible translocated zooxanthellar acid found in the muscle tissues of *T. maxima* as this acid is within 2‰ of the zooxanthellar fatty acid $\delta^{13}\text{C}_{20:5\omega3}$. This fatty acid, however, has a quite different $\delta^{13}\text{C}_{20:5\omega3}$ when present in *T. maxima* mantle tissues and resembles the isotopic composition of POM $\delta^{13}\text{C}_{20:5\omega3}$.

very closely (within 2‰) A noteworthy feature of the isotopic data is the ^{13}C -depleted value of the 18:3 ω 6 fatty acid in mantle and digestive mass tissues compared to the $\delta^{13}\text{C}_{16:0}$, a feature also apparent in the tissues of *T. gigas* (section 3.8.2). This ^{13}C -depletion (circa 15‰) cannot be explained by isotopic effects involved in fatty acid elongation and desaturation transformations from zooxanthellar acids and must reflect a predominant POM source, although this acid is absent in the particular POM sampling taken for this study. Why this particular fatty acid should have such a ^{13}C -depleted isotopic value is unknown. Other predominant mantle (18:1 ω 9, 18:4, 18:5, 20:4 ω 6 and 22:6 ω 3), muscle (18:0, 18:1 ω 9, 20:5 ω 3, 22:6 ω 3) and digestive mass (18:0, 18:5, 20:5 ω 3, 22:6 ω 3) fatty acids which also occur in zooxanthellae, appear to have isotopic compositions that are >2‰ ^{13}C -depleted or ^{13}C -enriched compared to the zooxanthellar $\delta^{13}\text{C}$ fatty acid compositions. The evidence that these acids are isotopically >2‰ different to zooxanthellar $\delta^{13}\text{C}$ values does not preclude those acids being translocated to host tissues but appears to provide a measure, in terms of the relative proportion of each fatty acid, of the importance of either zooxanthellae or filter feeding sources. Apparently for the animal TM1; 16:0, 16:1 ω 7 and 18:0 are fatty acids that are predominantly sourced from the zooxanthellae.

3.8.2 *T. gigas* - mantle, muscle, gills and digestive mass tissues

Unfortunately isotopic data for individual fatty acids in POM from Pioneer Bay and JCU aquarium are sparse. POM from One Tree Lagoon and published values for phyto-, zooplankton and dissolved organic matter, generally possess ^{13}C -depleted acid compositions (section 3.5.3), compared to whole-body and individual tissue values in *T. maxima*. It is thus assumed that POM from Orpheus and the JCU aquarium will also have similar ^{13}C -depleted fatty acid $\delta^{13}\text{C}$ compositions compared to *T. gigas* tissues.

TG1

In Animal TG1 (collected from Pioneer Bay, Orpheus Island) the compositions of $\delta^{13}\text{C}_{14:0}$ and $\delta^{13}\text{C}_{16:0}$ in mantle, gill and digestive mass tissues are within 1‰ of $\delta^{13}\text{C}_{14:0}$ and $\delta^{13}\text{C}_{16:0}$ in isolated zooxanthellae (Fig.3.30). Furthermore the fatty acid 18:4 in TG1 digestive mass

tissues has an $\delta^{13}\text{C}_{18:4}$ isotopic composition within 2‰ of zooxanthellar $\delta^{13}\text{C}_{18:4}$. Other major fatty acids in mantle, gills and digestive mass tissues tend to be ^{13}C -depleted compared to the same fatty acids in isolated zooxanthellae and probably reflect a greater dependence on POM fatty acids than in *T. maxima*. This is a possible reflection of the more oligotrophic nature of One Tree Lagoon waters compared to Pioneer Bay.

TG2

The $\delta^{13}\text{C}_{16:0}$ in mantle, gills and digestive mass tissues, the $\delta^{13}\text{C}_{16:1\omega7}$ in mantle and muscle and the $\delta^{13}\text{C}_{22:6\omega3}$ in gill tissues of TG2 were within 1‰ of zooxanthellar 16:0 and 16:1 ω 7 and 22:6 ω 3 fatty acid isotopic compositions and are strong candidates for translocated fatty acids utilised directly by the host clam (Fig.3.31). As in animal TG1 other fatty acids are more ^{13}C -depleted than the $\delta^{13}\text{C}$ of zooxanthellar fatty acids and appear to be sourced from POM or a mixture of zooxanthellar and POM sources. Apparently for the zooxanthellate *T. gigas* animals (TG1 and TG2); 14:0, 16:0, 16:1 ω 7, and possibly the fatty acid 22:6 ω 3 in animal TG2 gills, are fatty acids that are predominantly sourced from the zooxanthellae.

TG3

The $\delta^{13}\text{C}$ isotopic profile for fatty acids isolated from the albino animal (TG3) is quite different to its fully zooxanthellate contemporaries. Fatty acids greater than 18 carbon lengths were not found in zooxanthellae isolated from the albino animal. From the profiles it appears that zooxanthellar 14:0, 16:0 are translocated directly to the host, as the values are within 1‰ of zooxanthellar $\delta^{13}\text{C}_{14:0}$ and $\delta^{13}\text{C}_{16:0}$ (Fig.3.32). The fatty acid 18:4 is also a possible translocated fatty acid as $\delta^{13}\text{C}_{18:4}$ in TG3 muscle is within 2‰ of zooxanthellar $\delta^{13}\text{C}_{18:4}$. Other tissue (mantle and muscle) fatty acids are enriched in ^{13}C compared to the zooxanthellar fatty acid isotopic compositions. Apparently for the partial albino *T. gigas* animal (TG3); 14:0, 16:0, 18:4 are fatty acids that are predominantly sourced from the zooxanthellae regardless of the much reduced numbers of zooxanthellae in this animal.

3.9 SUMMARY

Several deductions can be made concerning the translocation of fatty acids in *Tridacna*.

- (a) In *Tridacna* sp. a similar pattern of fatty acid translocation is apparent in *T. maxima* and *T. gigas*, based on comparison of carbon isotopic compositions of fatty acids in symbiotic bivalve tissues compared with zooxanthellar fatty acids (3.12).
- (b) The carbon isotopic composition of short chain fatty acids in zooxanthellae from *T. maxima* and zooxanthellae from *T. gigas* (including the partial albino animal TG3) are within ca. 1‰ for the fatty acids 14:0 and 16:0, and within ca. 2‰ for the fatty acids 16:1 ω 7 in both species (Fig.3.33). The isotopic similarity in these short chain acids feasibly suggests a common zooxanthellar biosynthesis method for both species.
- (c) Carbon isotopic compositions of zooxanthellar fatty acids suggest synthesis from acetate, short-cutting the carbon isotopic fractionation associated with the enzyme pyruvate dehydrogenase in the conversion of pyruvate to acetyl-CoA.
- (d) The ^{13}C -enriched values of $\delta^{13}\text{C}_{\text{zooxanthellae}}$ may be indicative of carbon limitation.

Table 3.12 Major translocated fatty acids in the species *Tridacna*, based on comparison on $\delta^{13}\text{C}$ of tissue fatty acids compared with zooxanthellar fatty acids.

Species, tissue type	Fatty acids within 1‰ $\delta^{13}\text{C}$ of zooxanthellar fatty acid $\delta^{13}\text{C}$	Fatty acids within 2‰ $\delta^{13}\text{C}$ of zooxanthellar fatty acid $\delta^{13}\text{C}$
<i>T. maxima</i> , whole-body (Encore)	16:1 ω 7, 18:0	16:0, 18:3 ω , 6 18:5, 20:5 ω 3
<i>T. maxima</i> , mantle (TM1)	16:1 ω 7	16:0, 18:0
<i>T. maxima</i> , muscle (TM1)	16:0	20:5 ω 3
<i>T. maxima</i> , digestive mass (TG1)	18:0	-
<i>T. gigas</i> , mantle (TG1)	14:0, 16:0	-
<i>T. gigas</i> , gills (TG1)	16:0	-
<i>T. gigas</i> , digestive mass (TG1)	16:0	18:4
<i>T. gigas</i> , mantle (TG2)	16:0, 16:1 ω 7	-
<i>T. gigas</i> , muscle (TG2)	16:1 ω 7	16:0
<i>T. gigas</i> , gills (TG2)	16:0, 22:6 ω 3	-
<i>T. gigas</i> , digestive mass (TG2)	16:0	20:5 ω 3
<i>T. gigas</i> , bleached mantle (TG3)	16:0	-
<i>T. gigas</i> , normal mantle (TG3)	14:0	18:4
<i>T. gigas</i> , muscle (TG3)	-	16:0, 18:4

3.10 LIPIDS IN NON-SYMBIOTIC BIVALVES.

3.10.1 Total lipids

Lipids were analysed from nine non-symbiotic bivalves, collected from One Tree Lagoon, to enable a comparison to be made with the lipid compositions of symbiotic bivalves. The total lipid concentrations of the non-symbiotic bivalve tissues are given in Table 3.13.

A standard deviation is only reported on the lipid composition of *S. cucculata* as the majority of species were represented by less than three analyses, the number of individuals is indicated in parenthesis in Table 3.13. Whole-body total lipid analysis was carried out on two non-symbiotic species, 29 individuals of the species *S. cucculata* and 3 of the species *S. bilocularis*. Total lipid compositions of the species *S. bilocularis* were 18.5% and 3.9% respectively from animals for two different sites (2 animals adjacent to OTI and one animal from one of the patch reefs in the One Tree lagoon).

Table 3.13. Total lipid concentrations in non-symbiotic bivalve tissues. Lipids expressed as percent lipids per dry weight of tissue extracted, values are average values when more than one individual was analysed. Number in parenthesis refers to the number of individuals analysed.

Species (non-symbiotic)	Whole-body	Adductor muscle	Mantle	Combined remaining tissues (CRT)
<i>S. squamosus</i>	-	0.9 (2)	3.3 (2)	4.4 (2)
<i>A. reticulata</i>	-	1.7 (1)	1.6 (1)	4.9 (1)
<i>S. bilocularis</i> , site 1	18.5 (2)	-	-	-
<i>S. bilocularis</i> , site 2	3.9 (1)	-	-	-
<i>P. bicolor</i>	-	2.6 (1)	8.6 (1)	4.6 (1)
<i>B. foliata</i>	-	4.6 (1)	1.3 (1)	15.8 (1)
<i>P. margaritifera</i>	-	2.1 (2)	4.7 (2)	5.8 (2)
<i>S. cucullata</i>	8.9±6.8 (29)	-	-	-
<i>H. hyotis</i>	-	3.7 (2)	4.9 (2)	6.6 (2)
<i>M. regula</i>	-	1.4 (1)	5.7 (1)	14.5 (1)

The average lipid composition for the species *S. cucullata*, was 8.9±6.8%, a level very similar to that reported in section 3.7.2 for symbiotic bivalve whole-body tissues. The standard deviation is high because three of the animals had anomalously high lipid contents compared with their contemporaries, at 17.6, 22.5 and 39.7%. If these three animals are not included in the statistics the average lipid content becomes 7.1±2.3%. It is not clear why these three particular animals had higher lipid contents as they were all sampled from the same population. A weak positive correlation between animal size (in terms of shell dimensions) and lipid content was found for 21 out of 23 animals; however, animals containing the highest lipid contents of 22.5 and 39.7% do not fall on this regression line (Fig.3.34). No significant difference was found in the fatty acid compositions of these two anomalous animals. The lipid composition of adductor muscle, mantle and combined remaining tissues ranges from 0.9-4.6%, 1.3-8.6% and 4.4-15.8% respectively in seven non-symbiotic bivalves. Lipid levels are very similar to symbiotic bivalve tissues for muscle and combined remaining tissues (section 3.7.2), however, the range for mantle tissue of ca. 1-9% was larger than that for symbiotic

animals (6-8%). Generally, however, lipid levels in isolation cannot be taken to provide evidence for the presence of zooxanthellae within the tissues of *Tridacna*.

3.10.2 Free fatty acid compositions of whole-body tissues of non-symbiotic bivalves

Saccostrea cucullata:

Twenty-one individuals of this species were analysed as whole-body tissues. Saturated fatty acids formed circa 50% on average of the total fatty acids in *S. cucullata*. The average unsaturation index for *S. cucullata* was 140 and the $\omega 6/\omega 3$ ratio was 0.4. The most predominant fatty acids found were 16:0, 18:0, 20:1 and 22:6 $\omega 3$ (in order of abundance), these fatty acids forming just over 50% of the total fatty acids. Variability in percentage values for these fatty acids was high at approximately 20% but fatty acid profiles remained similar with the four fatty acids mentioned always being the most predominant and always in the order above (Fig. 3.35).

Septifer bilocularis.

Three individuals of this species were analysed from two sites in One Tree lagoon, site 1 was located adjacent to One Tree Island and site 2 was a small micro-atoll in the lagoon (micro-atolls 1-12 were locations where *T. maxima* and POM were sourced for this study). Saturated fatty acids formed between 42 and 46% of the total fatty acids in *S. bilocularis*. The unsaturation indices and $\omega 6/\omega 3$ ratios for the whole-body tissues of *S. bilocularis* from sites 1 and 2 were 144 and 155 and 0.4 and 0.8 respectively. The most predominant fatty acids for the animal collected from site 1 were 16:0, 16:1 $\omega 7$, 18:0 and 22:6 $\omega 3$ and for the animal from site 2 the most abundant fatty acids were 16:0, 18:0, 22:6 $\omega 3$ and 20:1 (Fig.3.36).

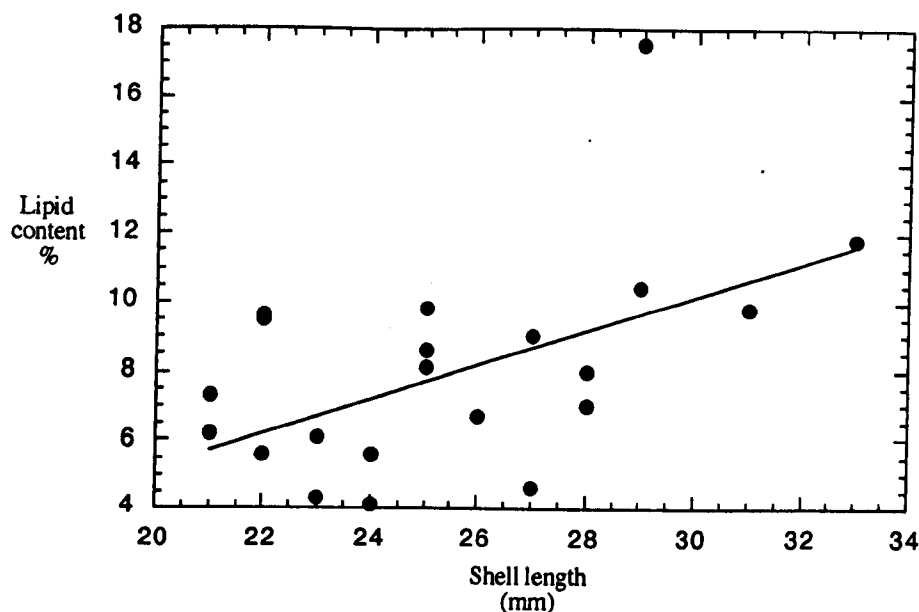


Fig. 3.34 Shell length versus lipid content in the species *S. cucullata* (Regression curve of $y = -4.54 + 0.49x$, $R = 0.517$)

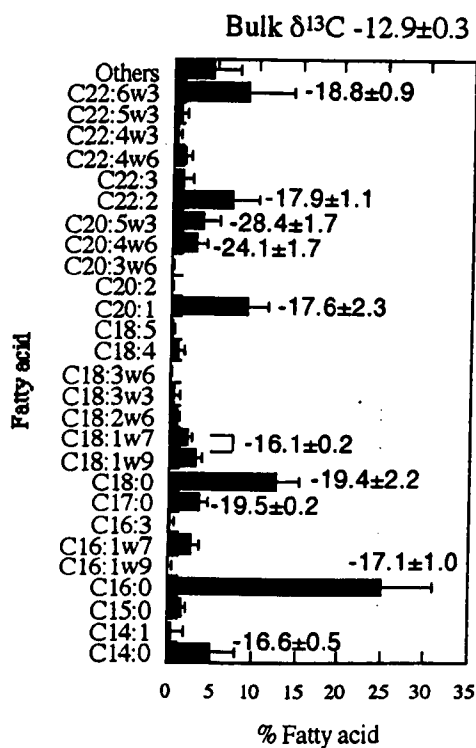


Fig. 3.35 Fatty acid and isotopic profile for whole body tissues in the non symbiotic bivalve *Saccostrea cucullata*. % acids represent average values from 21 individuals. Isotopic data represent average values from two animals, ‰, errors 1 σ .

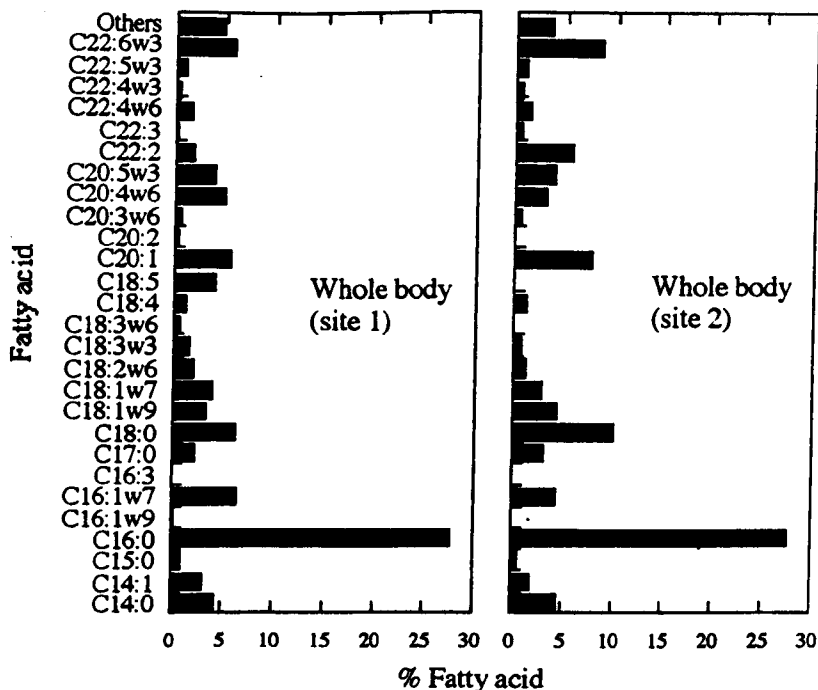


Fig. 3.36 Fatty acid profiles for whole body tissues in the non symbiotic bivalve *Septifer bilocularis*. Values from 2 individuals collected from different locations (sites 1 and 2).

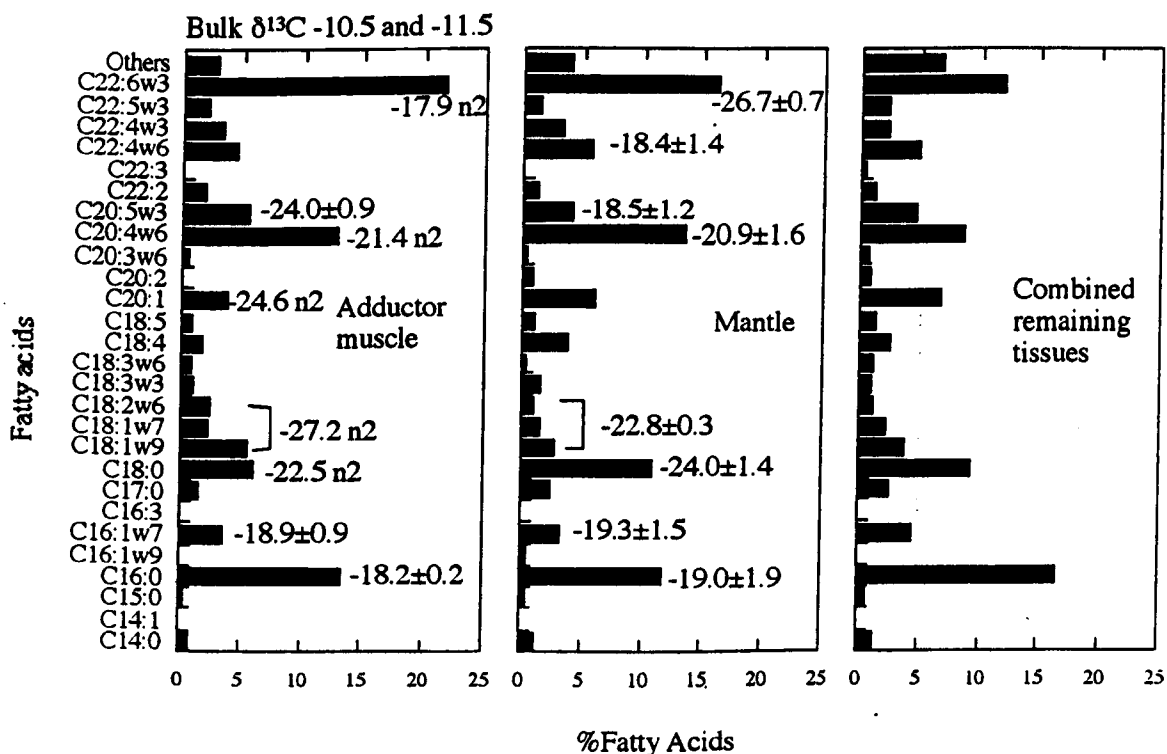


Fig. 3.37 Fatty acid and isotopic profiles for the tissues, adductor, mantle and combined remaining tissues in the non symbiotic bivalve *Spondylus squamosus*, collected from OT lagoon. Average values from 2 individuals. (‰PDB, errors 1σ)

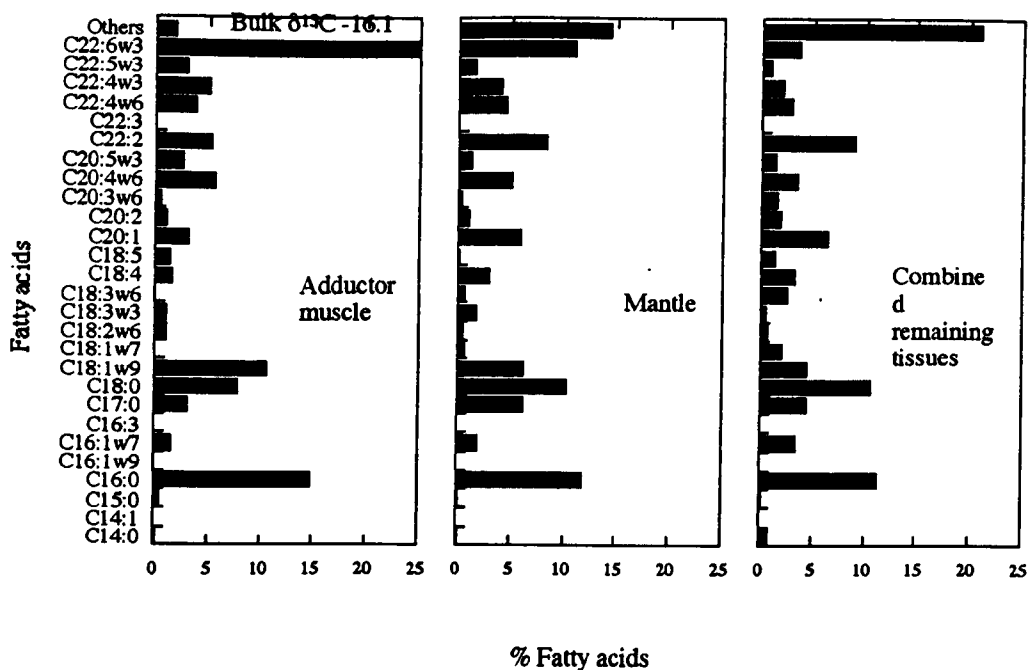


Fig. 3.38 Fatty acid profiles for the tissues, adductor, mantle, combined remaining tissues in the non symbiotic bivalve *Antigona reticulata*. Values from 1 individual.

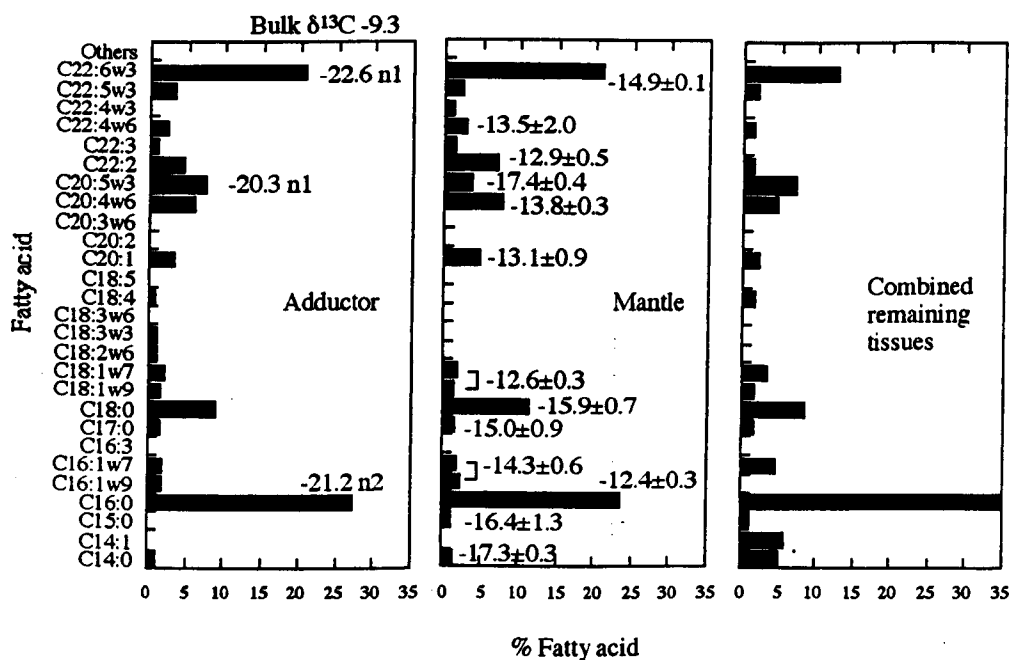


Fig. 3.39 Fatty acid and isotopic profiles for the tissues, adductor, mantle and combined remaining tissues in the non symbiotic bivalve *Pinna bicolor*. Values from 1 individual (% , errors 1 σ).

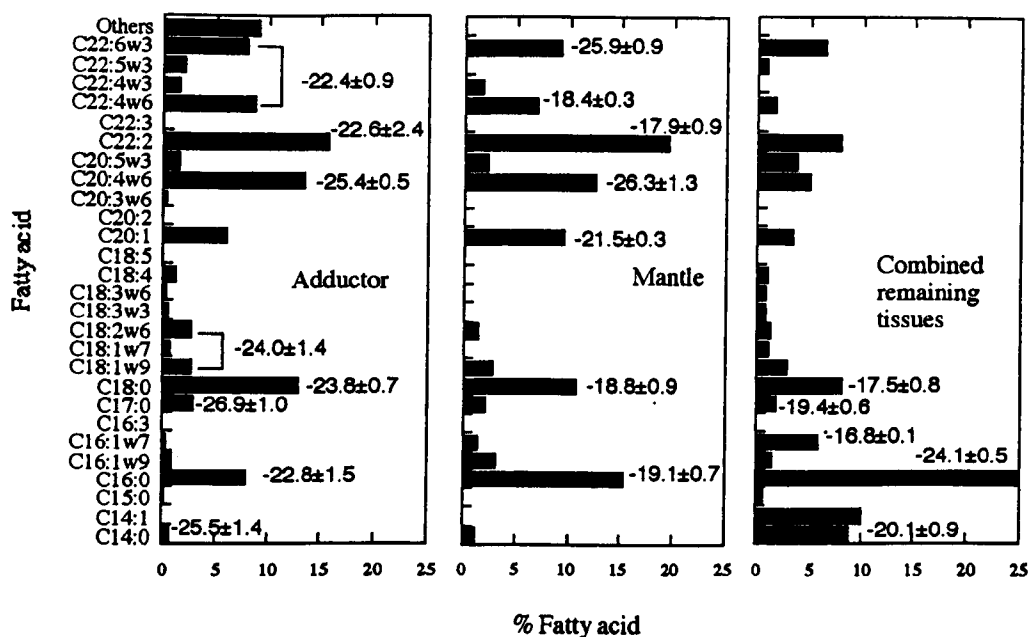


Fig. 3.40 Fatty acid and isotopic profiles for the tissues adductor, mantle and combined remaining tissues in the non symbiotic bivalve *Barbatia foliata*, collected from OT lagoon. Values from 1 individual (‰PDB, errors 1σ.).

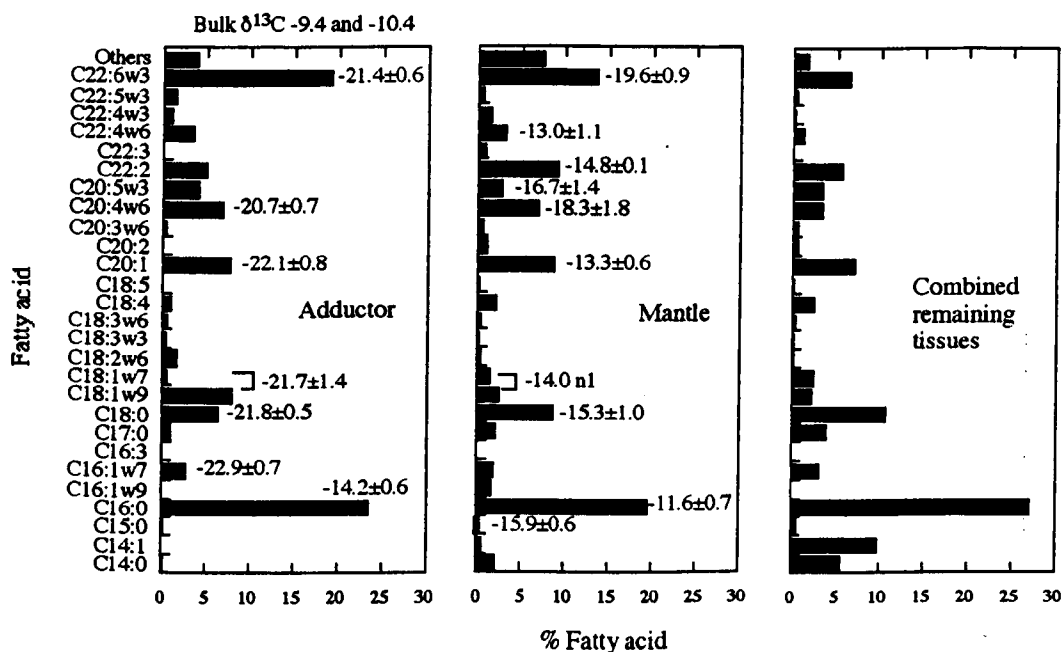


Fig. 3.41 Fatty acid and isotopic composition of adductor, mantle and combined remaining tissues in the non symbiotic bivalve *Pinctada margaritifera*. Values from 2 individuals, collected from OT lagoon. (‰PDB, errors 1σ.).

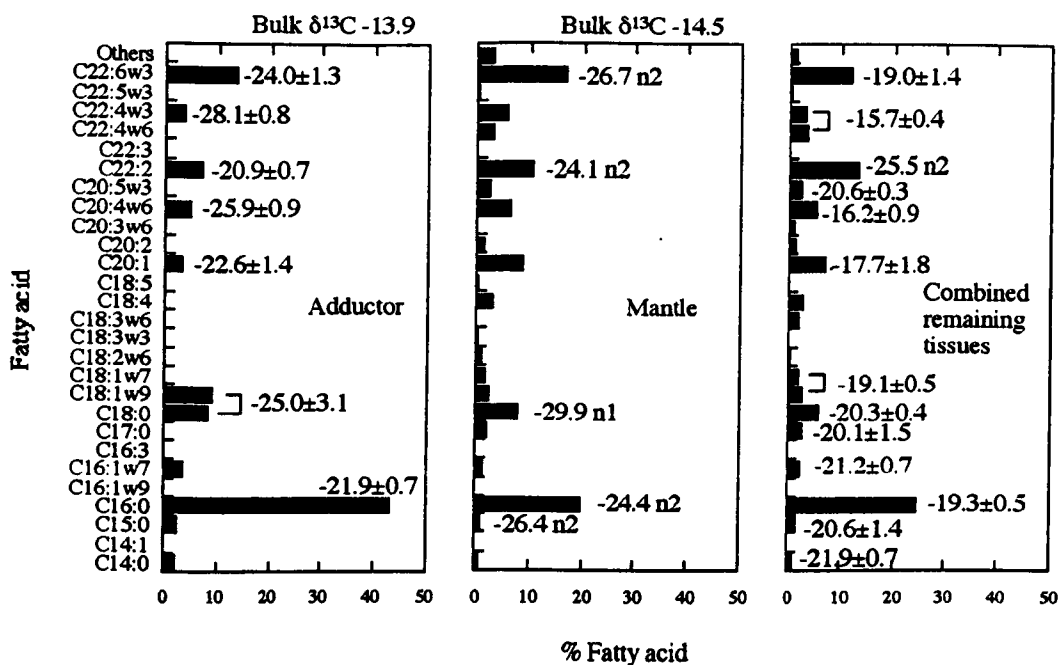


Fig. 3.42 Fatty acid and isotopic profiles for the tissues adductor, mantle and combined remaining tissues in the non symbiotic bivalve *Hyotissa hyotis*. Values from 2 individuals. (‰PDB, errors 1 σ .)

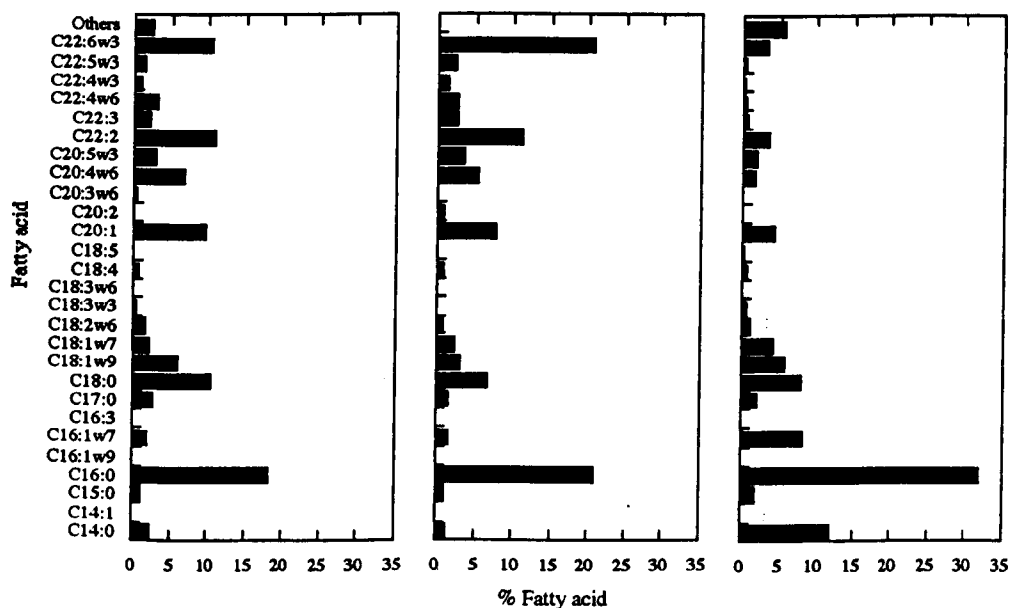


Fig. 3.43 Fatty acid profiles for the tissues, adductor, mantle, combined remaining tissues in the non symbiotic bivalve *Malleus regula*. Values from 1 individual.

3.10.3 Fatty acid compositions of individual tissues of non-symbiotic bivalves

The saturated fatty acid content, unsaturation indices and $\omega 6/\omega 3$ ratios for muscle, mantle and combined remaining tissues (CRT) from the non-symbiotic bivalves are given in Table 3.14. Average $\omega 6/\omega 3$ ratios of 0.6, 0.7 and 0.7 were found for adductor muscle, mantle and CRT tissues. Non-symbiotic whole-body $\omega 6/\omega 3$ ratios were lower at 0.5.

Spondylus squamosus:

The four most abundant fatty acids in muscle, mantle and CRT tissues were 22:6 ω 3, 16:0, 20:4 ω 6 and 18:0 and these fatty acids form circa 50% of all fatty acids in all three tissues (Fig. 3.37). The order of fatty acid abundance is different for each tissue type. The PUFA 22:6 ω 3 was the predominant fatty acid in adductor muscle and mantle tissues and formed >15% and >20% of the total fatty acids in these tissues respectively. Although 22:6 ω 3 was also in abundant in CRT tissues the most predominant fatty acid in CRT was 16:0 which formed >15% of the total fatty acid pool.

Table 3.14 Saturated fatty acid content, unsaturation indices and $\omega 6/\omega 3$ ratios for muscle, mantle and combined remaining tissues (CRT) from the non-symbiotic bivalves *S. squamosus*, *A. reticulata*, *P. bicolor*, *B. foliata*, *P. margaritifera*, *H. hyotis*, and *M. regula*.

Species / tissue type	Saturated fatty acids as % of total	Unsaturation index (UI)	$\omega 6/\omega 3$ fatty acid ratio
<i>S. squamosus</i> / muscle	22.1	295	0.6
<i>S. squamosus</i> / mantle	27.0	263	0.8
<i>S. squamosus</i> / CRT	30.5	221	0.7
<i>A. reticulata</i> / muscle	26.1	282	0.3
<i>A. reticulata</i> / mantle	28.5	188	0.6
<i>A. reticulata</i> / CRT	27.3	142	1.3
<i>P. bicolor</i> / muscle	39.2	247	0.3
<i>P. bicolor</i> / mantle	39.3	237	0.4
<i>P. bicolor</i> / CRT	51.5	172	0.3
<i>B. foliata</i> / muscle	24.7	217	1.8
<i>B. foliata</i> / mantle	29.4	210	1.6
<i>B. foliata</i> / CRT	44.6	143	0.7
<i>P. margaritifera</i> / muscle	31.4	230	0.5
<i>P. margaritifera</i> / mantle	32.3	197	0.6
<i>P. margaritifera</i> / CRT	45.9	126	0.5
<i>H. hyotis</i> / muscle	55.9	143	0.3
<i>H. hyotis</i> / mantle	31.1	229	0.4
<i>H. hyotis</i> / CRT	36.6	197	0.7
<i>M. regula</i> / muscle	31.9	246	0.3
<i>M. regula</i> / mantle	35.0	188	0.7
<i>M. regula</i> / CRT	55.9	84	0.5

Antigona reticulata:

The most predominant fatty acids in the adductor muscle, mantle and CRT tissues of this species were 16:0, 22:6 ω 3, 18:0, 18:1 ω 9, 22:2 and 20:1 (Fig. 3.38). Adductor muscle was particularly rich in 22:6 ω 3 (ca.25% of total fatty acids). The abundance of fatty acids other than the 27 fatty acids listed in each fatty acid profile was high in the CRT of this species.

Pinna bicolor:

The fatty acid profiles for adductor muscle, mantle and CRT from this species were all similar in their content and the three most abundant fatty acids are the same for all three tissues; the fatty acids 16:0, 22:6 ω 3 and 18:0 (Fig. 3.39).

Barbatia foliata:

The fatty acid profiles for the three tissues in this species were quite different. In adductor muscle the fatty acids 22:2, 20:4 ω 6, 18:0 and 22:4 ω 6 were the most abundant. In contrast predominant mantle tissue fatty acids were 22:2, 16:0, 20:4 ω 6 and 18:0 and the most predominant fatty acids in CRT tissues were 16:0, 14:1, 14:0 and 18:0 (Fig. 3.40)

Pinctada margaritifera:

The most abundant fatty acids in this species were 16:0, 22:6 ω 3, 18:1 ω 9 and 20:1 in adductor muscle, 16:0, 22:6 ω 3, 22:2 and 20:1 in mantle tissue and predominant CRT fatty acids were 16:0, 22:2, 22:6 ω 3 and 20:1 (Fig. 3.41).

Hyotissa hyotis:

The most abundant fatty acids (in order of abundance) in the adductor muscle of *H. hyotis* were 16:0, 22:6 ω 3, 18:1 ω 9 and 18:0. The fatty acids 16:0, 22:6 ω 3, 22:2 and 20:1 predominated the fatty acid profiles in mantle tissue and CRT (Fig. 3.42).

Malleus regula:

The most abundant fatty acids in this species were 16:0, 22:2, 22:6 ω 3 and 18:0 in adductor muscle, 16:0, 22:6 ω 3, 22:2 and 20:1 in mantle tissue. In contrast the most predominant fatty acids in CRT were 16:0, 14:0, 16:1 ω 7 and 18:0 (which formed 60% of total), (Fig.3.43).

3.10.4 Free fatty acid abundances in non-symbiotic bivalves –an overview

Saturated fatty acids.

The major saturated fatty acid in all of the non-symbiotic bivalves analysed was 16:0, and in the majority of species 16:0 was the most predominant of all total fatty acids. The fatty acid 16:0 formed around 25-30% of total fatty acids in whole-body analyses in the two species *Saccostrea cucullata* and *Septifer bilocularis* and 8.0-43.1% in adductor muscle, 11.7-23.5% in mantle tissue, 11.4-34.9% in combined remaining tissues of the bivalves *S. squamosus*, *A. reticulata*, *P. bicolor*, *B. foliata*, *P. margaritifera*, *H. hyotis* and *M. regula*. The fatty acid, 14:0, was also a significant component in the whole-body tissues of the bivalves *S. cucullata* and *S. bilocularis* (5.2% and 4.5% respectively) and in the combined remaining tissues of *P. bicolor*, *B. foliata*, *P. margaritifera* and *M. regula* at 5.2-12.0%. Levels of 14:0 in adductor muscle and mantle tissues were typically lower at 0.2-2.4% for all non-symbiotic species. The odd chain saturated fatty acids 15:0 and 17:0 were present in low abundance in individual tissues from most of the non-symbiotic bivalves. Whole-body tissues 15:0 and 17:0 levels were 0.7-1.6% and 2.3-3.6% respectively in the species *S. bilocularis* and *S. cucullata*. Mantle 15:0 and 17:0 levels were 0.0-1.3% and 1.8-2.5% and adductor muscle levels 0-2.5% and 0-3.0% respectively for the species *S. squamosus*, *P. bicolor*, *B. foliata*, *P. margaritifera*, *H. hyotis* and *M. regula*. The species *A. reticulata* was unusual in that it possessed less than 0.5% of the fatty acid 15:0 in its tissues but higher amounts of 17:0 than the other non-symbiotic bivalves (3.1-6.2%). The reason for the higher concentration of the fatty acid 17:0 is unclear; however, odd chain fatty acids have been reported previously in non-symbiotic molluscs (Dunstan *et al* 1992), but their presence is often indicative of bacterial input, either from bacterial contamination of the samples, or a dietary source containing a bacterial component. Bacteria may be a significant component of the particulate organic matter in the waters of One Tree lagoon as odd chain fatty acids were also found in low abundance in the tissues of *Tridacna* species. The highest concentrations of odd chain fatty acids were generally found in the combined remaining tissues, of non-symbiotic bivalves, which comprised the digestive

mass as one of its components, therefore it is feasible that the presence of these fatty acids is dietary in origin. The presence of the fatty acid 15:0 in three of the POM samples from One Tree Lagoon is additional evidence for a bacterial dietary component.

Mono-unsaturated fatty acids

Of the monounsaturated fatty acids identified, 20:1 was the most abundant at 3.1-7.9%, 4.9-9.7%, 2.1-7.1% and 4.9-9.7% in adductor muscle, mantle, combined remaining tissues and whole-body tissues of non-symbiotic species. This fatty acid comprised 3 isomers though the positions of the double bonds are unknown. Both 16-carbon and 18-carbon mono-unsaturated fatty acids, were represented by two isomers, omega9 and omega7 in non-symbiotic bivalves. The fatty acid 16:1ω9 was either absent or found in very low concentrations (< 3%) in all tissues (adductor muscle, mantle and CRT) from non-symbiotic bivalves. The fatty acid 16:1ω7 was present in all non-symbiotic bivalve tissues and formed 0.4-3.6%, 1.5-3.3%, 2.3-8.2% and 1.5-3.3% of the total fatty acids in adductor muscle, mantle, CRT and whole-body tissues respectively. In contrast the omega 9 isomer, 18:1ω9, was the most abundant of the two 18-carbon mono-unsaturated fatty acids. The fatty acid, 18:1ω9, formed 1.8-10.6%, 1.4-6.2%, 1.8-5.8%, 1.4-6.2% of the total fatty acids in adductor muscle, mantle, CRT and whole-body tissues in non-symbiotic species whereas the fatty acid, 18:1ω7, typically formed <4% of all total fatty acids in all tissues from all non-symbiotic species.

Polyunsaturated fatty acids

Polyunsaturated fatty acids comprised just under one third of the total fatty acids in the whole-body tissues of the species *S. cucullata* and *S. bilocularis*, 31.4-59.9% in adductor muscle tissue, 42.7-54.7% in mantle and 15.6-47.7% in the combined remaining tissues in the species *S. squamosus*, *A. reticulata*, *P. bicolor*, *B. foliata*, *P. margaritifera*, *H. hyotis* and *M. regula*. Unsaturation indices for non-symbiotic species ranged from 140-155 in whole-body tissues, 143-295 in adductor muscle, 188-263 in mantle and 84-221 in combined remaining tissues. These indices are very similar to those found in particulate organic matter (93-278) from the

One Tree lagoon (section 3.5.2). Polyunsaturated fatty acids with 18-, 20- and 22-carbon chains were all detected in the non-symbiotic bivalve tissues. Eighteen carbon chain fatty acids 18:2 ω 6, 18:3 ω 3, 18:4 and 18:5 occurred in low concentrations, typically less than 4%, in all tissues (adductor muscle, mantle and CRT) in all of the non-symbiotic bivalves analysed. Fatty acids 20:2 and 20:3 ω 6 were either absent or present in low abundance (< 2%) in non-symbiotic bivalves. The fatty acids 20:4 ω 6 and 20:5 ω 3, present in concentrations of 1.8-13.5% and 0.0-7.5% respectively, were found in the majority of tissues from all non-symbiotic bivalves. Polyunsaturated 22-carbon fatty acids with 2-6 double bonds were also found. The fatty acid 22:2 was found in all tissues from all non-symbiotic bivalves and was represented by two isomers, the position of their double bonds is unknown, total 22:2 amounted to 1.2-19.4% of total fatty acids in all tissues from non-symbiotic bivalves. A minor component, the fatty acid 22:3, formed 0.2-1.1% of the total fatty acids in whole-body tissues and < 3% in adductor muscle, mantle and combined remaining tissues of non-symbiotic bivalves. The PUFAs 22:4 ω 6 and 22:4 ω 3, were present in concentrations of 0-8.6% and 0-5.4% in non-symbiotic tissues (adductor muscle, mantle and CRT), 22:5 ω 3 was also a minor component of all non-symbiotic bivalve tissues at <3.5% of the total fatty acids. By far the most predominant PUFA in non-symbiotic bivalves was 22:6 ω 3, in whole-body tissues it amounted to 5.9-8.8% of the total fatty acids. Adductor muscles from the species *S. squamosus*, *A. reticulata*, *P. bicolor* and *M. regula* were particularly rich in this acid, often comprising 20% of the total fatty acids. Generally the ω 6/ ω 3 ratios in non-symbiotic animals are below 1 with the exception of mantle and muscle tissue from the species *B. foliata* and the combined remaining tissues of *A. reticulata*. At this point it is necessary to describe the habitats in which the non-symbiotic animals were found since this has bearing on following discussions.

Table 3.15 Habitats and attachment mechanisms of the non-symbiotic bivalves collected for this study from One Tree Island.

Species	Habitat	Attachment to substratum
<i>S. squamosus</i>	Coral rubble	Cemented
<i>S. cuccullata</i>	Coral rubble	Cemented
<i>H. hyotis</i>	Coral rubble	Cemented
<i>P. margaritifera</i>	Rock pool	Unclear as to attachment
<i>A. reticulata</i>	Sand	Burrowed
<i>B. foliata</i>	Coral rubble	Byssus
<i>P. bicolor</i>	Muddy sand	Burrowed
<i>M. regula</i>	Coral rubble	Byssus
<i>S. bilocularis</i>	Coral rubble	Byssus

Six of the nine non-symbiotic animals collected were attached to coral rubble, either cemented to the debris or wedged within gaps in the coral and held by a byssal strand. *A. reticulata* and *P. bicolor* were found partially buried in sand and are thus assumed to be burrowers. *P. margaritifera* was found in a rock pool at low tide. It appeared that this animal may have become stranded in this position as it was not obviously attached to the substratum. The base of the pool was sandy and the shape of the animal could indicate burrowing habitat or a free swimming form similar to that of scallops. The burrowing species do not appear to have fatty acids profiles which are markedly different to those attached to the coral, yet burrowers are able to source detrital material from the sand surfaces which are not readily available to animals attached high on the coral.

3.10.5 Bulk tissue $\delta^{13}\text{C}$ compositions in non-symbiotic bivalves from OTI.

$\delta^{13}\text{C}_{\text{whole-body}}$ values from the species *S. cucculata* were $-12.9 \pm 0.3\text{‰}$. $\delta^{13}\text{C}_{\text{muscle}}$ values ranged from -9.3‰ to -16.1‰ in the species *S. squamosus*, *A. reticulata*, *P. bicolor*, *P. margaritifera* and *H. hyotis*. Only one mantle tissue was analysed for the species *H. hyotis* that yielded a $\delta^{13}\text{C}$ value of -13.9‰ (Table 3.15). These isotopic compositions are very similar to those of symbiotic bivalves from the same site. On bulk carbon isotopic compositions alone it would not be possible to ascertain whether the symbiotic animals were indeed deriving the bulk of the nutrition from filter feeding or the symbiotic algae. (Fig. 3.44).

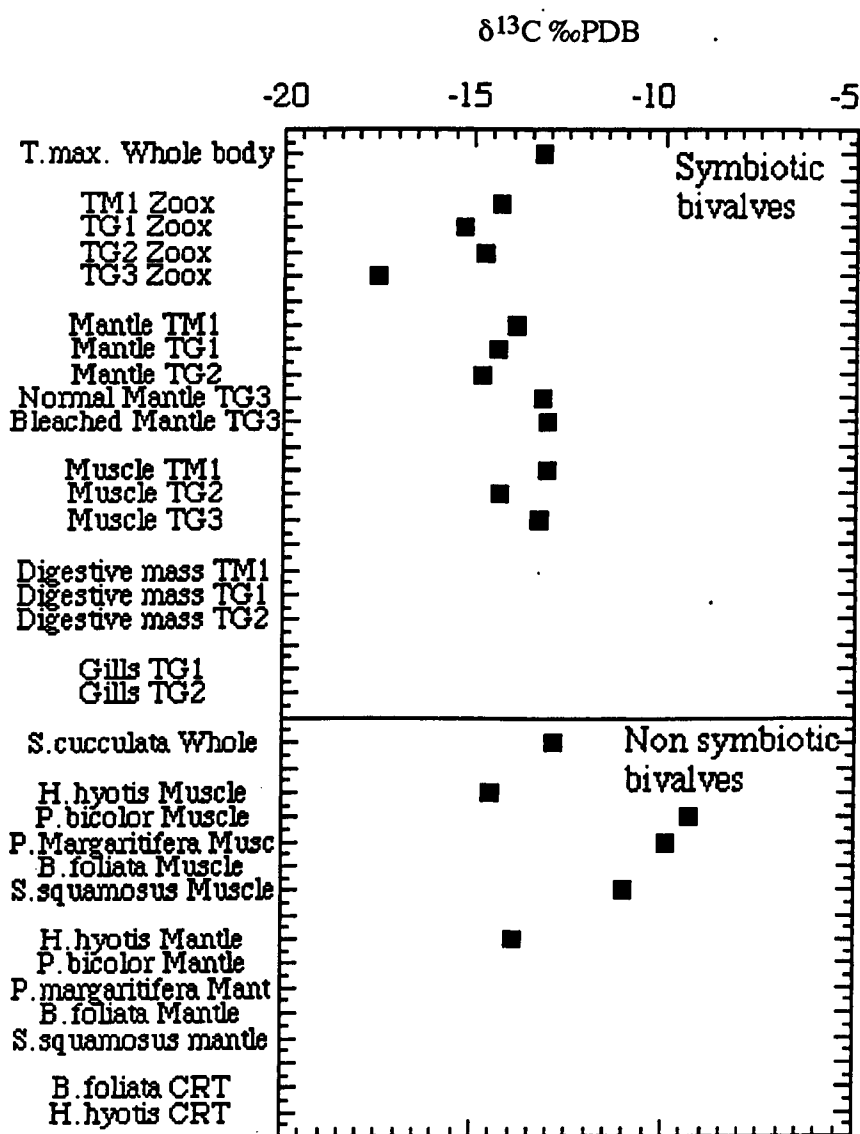


Fig. 3.44 Bulk $\delta^{13}\text{C}$ compositions of tissues from symbiotic and non-symbiotic bivalves.

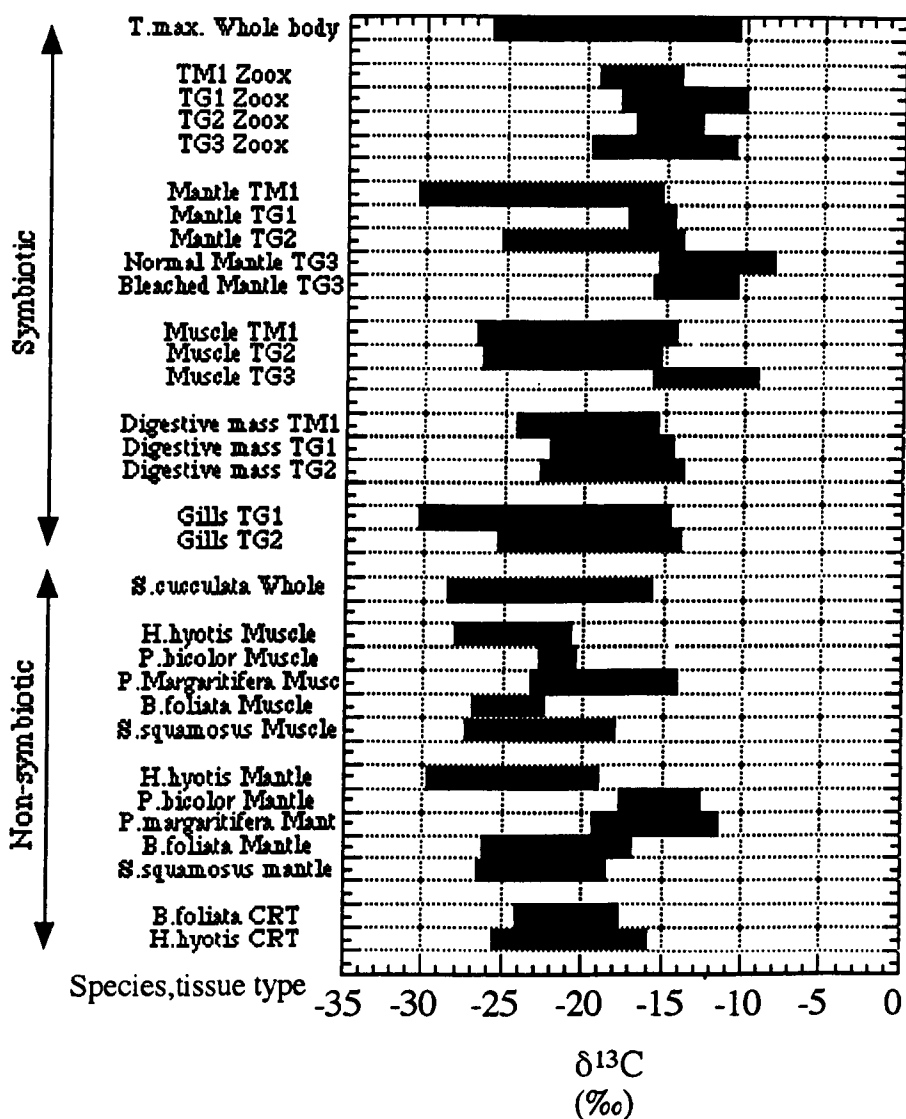


Fig. 3.45 $\delta^{13}\text{C}$ ranges of fatty acids isolated from symbiotic and non symbiotic bivalves

Table 3.16 Isotopic data for the non-symbiotic bivalves *H. hyotis*, *P. bicolor*, *P. margaritifera*, *B. foliata* and *S. squamosus*.

Species and Tissue type	$\delta^{13}\text{C}_{\text{tissue}}$ (‰)	$\delta^{13}\text{C}$ range of individual fatty acids (‰)	$\delta^{13}\text{C}_{16:0}$	$\Delta \delta^{13}\text{C}$ of all individual fatty acids relative to $\delta^{13}\text{C}_{16:0}$	$\Delta \delta^{13}\text{C}_{16:0}$ relative to $\delta^{13}\text{C}_{\text{tissue}}$ (‰)
<i>S. cucullata</i> , whole-body	-12.9±0.3	-16 to -28.4	-17.1 ± 1.0	+1.1 to -11.3	-4.2
<i>H. hyotis</i> , muscle	-14.5	-20.9 to -28.1	-21.9 ± 0.7	+1.0 to -6.2	-7.4
<i>H. hyotis</i> , mantle	-13.9	-24.1 to -29.9	-24.4 (n2)	+0.3 to -5.5	-10.5
<i>H. hyotis</i> , CRT	-	-15.7 to -25.5	-19.3 ± 0.5	+3.6 to -6.2	
<i>P. bicolor</i> , muscle	-9.3	-20.3 to -22.6	-21.2 (n2)	+0.9 to -1.4	-11.9
<i>P. bicolor</i> , mantle	-	-12.6 to -17.4	-12.4 ± 0.3	-0.2 to -5.0	
<i>P. margaritifera</i> , muscle	-9.9	-14.2 to -22.9	-14.2 ± 0.6	0 to -8.7	-4.3
<i>P. margaritifera</i> , mantle	-	-11.6 to -19.6	-11.6 ± 0.7	0 to -8.0	
<i>B. foliata</i> , muscle	-	-22.4 to -26.9	-22.8 ± 1.5	+0.4 to -4.1	
<i>B. foliata</i> , mantle	-	-17.9 to -26.3	-19.1 ± 0.7	+1.2 to -7.2	
<i>B. foliata</i> , CRT	-	-17.5 to -24.1	-24.1 ± 0.5	+6.6 to 0	
<i>S. squamosus</i> , muscle	-11.0	-17.9 to -27.2	-18.2 ± 0.2	+0.3 to -9.0	-7.2
<i>S. squamosus</i> , mantle	-	-18.4 to -26.7	-19.0 ± 1.9	+0.6 to -7.7	

3.10.6 Carbon isotopic compositions of whole-body and isolated tissues

Carbon isotopic analyses of individual fatty acids were carried out on the tissues from six non-symbiotic bivalves: *S. cucullata* (whole-body tissues), *H. hyotis* (muscle, mantle and combined remaining tissues), *P. bicolor* (muscle and mantle tissues), *P. margaritifera* (muscle and mantle tissues), *B. foliata* (muscle, mantle and combined remaining tissues) and *S. squamosus* (muscle and mantle tissues). Individual fatty acid carbon isotopic ranges for the various non-symbiotic tissues are given in Table 3.16. The individual fatty acid carbon isotopic compositions from the tissues of non-symbiotic bivalves were quite variable. The

total range of $\delta^{13}\text{C}_{\text{fatty acids}}$ for all species and all tissues from symbiotic bivalves was -11.6 to -29.9‰ and is very similar to the range in symbiotic bivalves of around -8 to -30‰ (Fig. 3.45). Isotopic compositions for several of the fatty acids from the species *P. bicolor* and *P. margaritifera* are generally more ^{13}C -enriched than for other non-symbiotic species. This presumably is a reflection of the use of an additional carbon source compared to their contemporaries, in the form of detrital material from the sand around them in their natural habitats. Saturated fatty acids are not always the most ^{13}C -enriched fatty acids in non-symbiotic bivalves and this presumably reflects diverse dietary sources for these animals. Saturated fatty acids from some non-symbiotic species yield isotopic values from -12 to -30‰. Without the full isotopic profile and phyto- and zoo-plankton composition of POM available for animals at One Tree Lagoon, integrated over a long time, it is impracticable to hypothesise on sources of these ^{13}C -enriched fatty acids in non-symbiotic algae. The ^{13}C -enriched fatty acids in non-symbiotic bivalves suggest that the biosynthesis of ^{13}C -enriched fatty acids may not be necessarily restricted to zooxanthellae.

3.10.7 Comparison of fatty acid data from symbiotic and non-symbiotic animals.

In comparing the fatty acid profiles of symbiotic versus non-symbiotic animals there is no single fatty acid that would provide a definitive marker for the presence of zooxanthellae within the symbiotic animals. However, the tissues from symbiotic species have significantly higher percentages of saturated fatty acids compared with the tissues from non-symbiotic bivalves ($t_{48}=2.94$, $P < .005$ at 95% confidence levels). (Statistical analyses of the data, in this study, were performed using the Microsoft Excel Analysis program. All t-test calculations were based on unpaired samples, two-tailed and assumed to have unequal variances). The implication of these results is that symbiotic bivalves may have a greater relative amount of saturated fatty acids than non-symbiotic bivalves because these saturated fatty acids are sourced from zooxanthellae. Therefore this implies that translocated fatty acids are predominately short chain saturated fatty acids, as longer than 18:0 saturated fatty acids are not present either in zooxanthellae or the tissues of symbiotic bivalves.

There are also significant differences in the means of fatty acid abundance between symbiotic and non-symbiotic bivalve tissues. The abundance means for the fatty acids 16:0, 16:1 ω 7, 17:0, 18:0, 18:1 ω 9, 18:1 ω 7, 18:3 ω 3, 18:3 ω 6, 18:4, 18:5, 20:1, 20:2, 20:3 ω 6, 22:2, 22:4 ω 3, are different for symbiotic versus non-symbiotic tissues (data in appendices). The fatty acids 16:0, 16:1 ω 7, 18:1 ω 9, 18:4, 18:5, 20:2 and 20:3 ω 6 are (more abundant in the tissues of symbiotic bivalves, whereas the fatty acids, 17:0, 18:0, 18:1 ω 7, 18:3 ω 3, 20:1 and 22:4 ω 3 are more abundant in non-symbiotic tissues. There is a close similarity between the fatty acids identified as predominant in the zooxanthellae isolated from *T. maxima*, *T. gigas* and *T. squamosa* (section 3.6.2) and between the fatty acids statistically identified as having higher means in symbiotic tissues. A significant difference exists in the ω 6/ ω 3 fatty acid ratios between symbiotic and non-symbiotic bivalves tissues ($t_{40}=3.393$, $P < .005$), 18:1 ω 9/18:1 ω 7 fatty acid ratios ($t_{34}=4.004$, $P < .005$) and 16:0/22:6 ω 3 fatty acid ratios ($t_{40}=3.739$, $P < .005$). The ratios ω 6/ ω 3, 18:1 ω 9/18:1 ω 7 and 16:0/22:6 ω 3 are consistently higher in symbiotic bivalve tissues and feasibly could be used as nutritional indicators of the symbiosis. The higher ω 6/ ω 3 ratios indicate, contrary to the fatty acid data in free living dinoflagellates, that the omega-6 fatty acids and not the omega-3 fatty acids predominate in the symbiotic association. The significance of this finding is important and may be indicative of fatty acid recycling within the symbiotic association.

There was a significant difference between the carbon isotopic values of two fatty acids in symbiotic compared to non-symbiotic acids: 16:0 ($t_{13}=3.778$, $P = < .005$) and 22:6 ω 3 ($t_{25}=3.649$, $P = < .005$). The mean isotopic compositions of both the fatty acids 16:0 and 22:6 ω 3 in symbiotic bivalves were +4.2‰ and +5.6‰ ^{13}C -enriched compared to $\delta^{13}\text{C}_{16:0}$ and $\delta^{13}\text{C}_{22:6\omega 3}$ in non-symbiotic tissues. For the majority of fatty acids, however, that were sufficiently abundant to provide isotopic analysis, there was no significant difference between the fatty acid carbon isotopic compositions between fatty acids isolated from non-symbiotic and symbiotic bivalves.

All non-symbiotic tissues analysed for bulk $\delta^{13}\text{C}_{\text{tissue}}$ had $\delta^{13}\text{C}_{16:0}$ compositions that were ^{13}C -depleted compared with bulk $\delta^{13}\text{C}_{\text{tissue}}$, with ^{13}C -depletions ranging from -4 to -12‰. The fatty acid $\delta^{13}\text{C}_{16:0}$ compositions in non-symbiotic are therefore yielding ^{13}C -depletions compared to $\delta^{13}\text{C}_{\text{tissue}}$ that are characteristic for the isotopic signatures of lipids compared to bulk tissue reported in the analysis of biochemical components (Nier and Gulbransen, 1939; DeNiro, 1977). In contrast, symbiotic tissues analysed for bulk $\delta^{13}\text{C}_{\text{tissue}}$ had $\delta^{13}\text{C}_{16:0}$ compositions that were only minimally ^{13}C -depleted compared with bulk $\delta^{13}\text{C}_{\text{tissue}}$ or were ^{13}C -enriched compared to bulk $\delta^{13}\text{C}_{\text{tissue}}$ (-2 to +3‰).

The isotopic difference of the 16:0 fatty acid from symbiotic and non-symbiotic confirms the hypothesis that this acid is an important form of translocated carbon for the species *Tridacna*. The data in this study suggest that this particular fatty acid is used immediately by the clam, in all tissues, and may also be the precursor for chain elongation and desaturation by the clam. The significance of this is that fatty acids in the symbiotic association may provide a reciprocal role. The 16:0 fatty acid translocated from the zooxanthellae to the clam may subsequently be transformed into a longer chain fatty acid by the clam (possibly desaturated, although bivalve desaturases are apparently less efficient (Langdon and Waldock 1981), which is subsequently translocated back to the zooxanthellae. This is one mechanism that could account for the ^{13}C -enriched fatty acids relative to 16:0 that are seen in *Tridacna*.

3.11 CONCLUSIONS

Several conclusions can be made from the analysis of lipids in symbiotic bivalves and the comparison of the data with that of lipids from non-symbiotic bivalves.

- (a) This chapter has provided a systematic analysis of the concentrations and isotopic compositions of the free fatty acids in the species *Tridacna* and has provided strong evidence for the translocation of lipids to the clam in the symbiotic association.
- (b) There is a significant difference between the carbon isotopic compositions of the fatty acids 16:0 and 22:6 ω 3 in symbiotic tissues compared with non-symbiotic tissues. As 16:0 is far more abundant in symbiotic bivalve tissues and has a consistently ^{13}C -enriched $\delta^{13}\text{C}_{16:0}$ compared to bulk $\delta^{13}\text{C}_{\text{tissue}}$ this is apparently conclusive evidence for the translocation of zooxanthellar 16:0 to the host clams.
- (c) Several other fatty acids have similar isotopic compositions to those same fatty acids in host tissues, which indicates direct translocation and utilisation of zooxanthellar fatty acids by the host in a variety of tissues or the recycling of fatty acids within the symbiotic association. Recycling and fatty acid chain transformations could account for the ^{13}C -enriched, long chain, desaturated fatty acids compared to $\delta^{13}\text{C}_{16:0}$.
- (d) Fatty acid isotopic compositions are only minimally ^{13}C -depleted or actually ^{13}C -enriched compared to bulk tissue $\delta^{13}\text{C}$ values, which suggests the synthesis of lipids from acetate, short-cutting the pyruvate to acetyl-CoA route and the accompanying isotopic fractionation associated with pyruvate dehydrogenase.
- (e) Lipid contents and fatty acid profiles in isolation cannot indicate the presence of photosymbiosis, although several fatty acids appear to be more abundant in symbiotic bivalve tissues. The ratios of ω 6/ ω 3, 18:1 ω 9/18:1 ω 7 and 16:0/22:6 ω 3 in symbiotic bivalves tissues indicate a different nutritional source to non-symbiotic bivalves, which may be related to the symbiotic algae.

(f) Bulk tissue carbon isotopic values are not a useful indicator in the *Tridacna* symbiosis as there is no significant difference between the isotopic compositions of symbiotic compared with non-symbiotic bivalves.

(g) The use of zooxanthellar translocated glucose by the host to synthesise 16:0 cannot be ruled out from the data presented in this chapter, as an isotopic composition for the glucose has not been determined. The use of translocated glucose as a starter for clam fatty acid synthesis would however involve the Calvin cycle and the subsequent utilisation of pyruvate dehydrogenase to provide acetyl-CoA for fatty acid chain synthesis. Provided the pool of pyruvate remained in excess of that converted to acetyl-CoA, the ^{13}C -discrimination exhibited by pyruvate dehydrogenase would be fully expressed, thus providing lipids that are ^{13}C -depleted compared to bulk $\delta^{13}\text{C}$ values. The average bulk $\delta^{13}\text{C}_{\text{zooxanthellae}}$ composition for zooxanthellae isolated from *T. maxima*, *T. gigas* and *T. squamosa* in this study was found to be -15.4‰. The carbohydrate component of an organism is typically +0.1‰ to +1.6‰ ^{13}C -enriched compared to the bulk $\delta^{13}\text{C}_{\text{tissue}}$ value (DeNiro, 1977). Feasibly the $\delta^{13}\text{C}_{\text{glucose}}$ in zooxanthellae would thus be -15.3‰ to -13.8‰, and 16:0 synthesised from this glucose would be typically be some 4-9‰ ^{13}C -depleted (DeNiro, 1977; Abelson and Hoering, 1961) compared to $\delta^{13}\text{C}_{\text{glucose}}$, yielding $\delta^{13}\text{C}_{16:0}$ compositions of between -18‰ and -23‰ if pyruvate is in excess of that converted to acetyl-CoA. The mean $\delta^{13}\text{C}_{16:0}$ composition in *T. maxima* and *T. gigas* was, however, -14.1‰. The only possible mechanism for synthesising $\delta^{13}\text{C}_{16:0}$ of -14.1‰ is by completely depleting the pyruvate pool in the conversion to acetyl-CoA and in this scenario there will be no isotopic fractionation and the 16:0 synthesised will have an identical isotopic composition to the glucose (-15.3 to -13.8‰). Clearly the translocation of glucose and the subsequent synthesis of fatty acids in a pyruvate-limited scenario merits further investigation.